THE INVOLVEMENT OF *PSEUDOMONAS PUTIDA* IN BASIDIOME INITIATION OF THE CULTIVATED MUSHROOM *AGARICUS BISPORUS*

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"Briefly, these poor bored fishermen played at first the ordinary children's games already established there long before; but afterwards when one of them invented a new game called 'pouring-from-the-empty-into-the-void,' they were all so pleased with it that thereafter they amused themselves with that alone"

ALL AND EVERYTHING

G. Gurdjieff

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ABBREVIATIONS

BATH	Bacterial adherence to hydrocarbons
CEA	Compost extract agar
cfu	Colony forming units
CMM	Compost malt medium
CSLM	Casing soil leachate medium
cy	Cyclopropane
EDDA	Ethylenediamine-*O*-hydroxyphenyl-acetic acid
EMS	Ethylmethane sulphonate
HGU	Hyphal growth unit
I.H.R.	Institute of Horticultural Research, Littlehampton, U.K.
ILU	Internode length unit
KA	King's medium A
KB	King's medium B
L.S.D.	Fisher's least significant difference test
MEA	Malt extract agar
MPA	Malt peptone agar
PAF	*Pseudomonas* agar F
PBS	Phosphate buffered saline
PDA	Potato dextrose agar
S.D.	Standard deviation
S.E.	Standard error of the mean
SAT	Salt aggregation test
SIL	Subapical internode length
TCH	Thiocarbohydrazide
TSBA	Tripticase soy broth agar
UV	Ultra-violet

ABSTRACT

The involvement of pseudomonads in the process of basidiome initiation of the cultivated mushroom *Agaricus bisporus* was investigated. Pseudomonads used throughout the study were identified and *Pseudomonas putida* was shown to be responsible for initiating basidiome morphogenesis. The prodigious morphogenetical capabilities of a single *P. putida* and *P. tolaasii* colony was demonstrated and the production of rough colonial forms in peat casing soil was observed. Rough colonial forms of *P. putida* were found to be capable of promoting basidiome initiation. Cells of the rough colonial forms were better able to withstand nutrient limited conditions, were more resistant to UV light, produced greater amounts of siderophore and respired at a faster rate than the smooth colonial variants which indicates that they are stress tolerant forms.

P. putida and *P. tolaasii* were shown to be positively attracted toward exudates of mushroom mycelium and adherence of these bacteria to hyphae was observed. Quantitative adherence studies, in conjunction with electron microscopy, revealed the ability of *P. putida* and *P. tolaasii* cells to adhere rapidly and firmly to hyphae. Differences in the chemotactic and adhesive abilities of smooth and rough colonial forms were observed.

A new medium for the growth of *A. bisporus* was developed and a strain of *A. bitorquis* W19 which produces primordia *in vitro*, when grown in association with basidiome stimulatory pseudomonads, was used as a model system for assaying the effect of bacteria on basidiome initiation. Activated charcoal was shown to replace the effects of *P. putida* and fruit body initiation did not to occur in response to nutrient limitation. Non-living *P. putida* failed to promote basidiome initiation and iron, and iron chelating agents did not stimulate fruiting. During the preliminary phase of the interaction between *P. putida* and *A. bisporus*, the bacterium was shown to markedly affect hyphal growth, colony morphology and the allocation of resources to the mycelium.

Mutants of *P. putida* defective in their ability to promote basidiome initiation were produced by transposon Tn*5* and UV light mutagenesis. The majority of these mutants were defective in

their ability to uptake the ferric-siderophore complex implicating the involvement of iron regulated, membrane bound protein porins in the process of fruit body initiation. It is thought that the mushroom mycelium produces a 'self-inhibitory-compound' which prevents basidiome morphogenesis until levels of this compound are reduced to below a threshold concentration. *P. putida* is thought facilitate this by importing the inhibitory substance into the cell, via the membrane bound protein porins, where it is subsequently metabolized.

A gene cloning and mapping system was developed in *P. putida* using RP4::mini-Mu. This plasmid was shown to promote transfer of auxotrophic markers in both homologous and heterologous matings and its potential as a gene cloning and mapping system in this organism was demonstrated.

CHAPTER ONE

GENERAL INTRODUCTION AND STUDY RATIONALE

1.1. GENERAL INTRODUCTION

Basidiome initiation of the cultivated mushroom, *Agaricus bisporus* (Lange) Imbach, is a complex and poorly understood phenomenon which occurs after mycelial colonization of a composted wheat straw/horse manure substrate. Basidiome development is stimulated by application of a layer of peat and lime (the 'casing' layer) to the surface of the colonized compost. A range of abiotic parameters including temperature, carbon dioxide concentration, humidity and pH, have been shown to markedly influence fruit body production (see Flegg & Wood 1985), but the 'key' to basidiome initiation is thought to be the presence of bacteria normally found in soil (Eger 1961, Wood 1976,), identified as *Pseudomonas putida* (Trevisan) Migula (Hayes, Randle & Last 1969, Rainey & Cole 1987). The exact effect of *P. putida* on the vegetative mushroom mycelium has yet to be determined, but it has been suggested that the mycelium produces 'self inhibitory compounds' which are removed by the bacteria to below a threshold level, permitting basidiome initiation to occur (Eger 1961, Long & Jacobs 1974, Wood 1976). The aim of this study was to investigate the involvement of *P. putida* in the process of basidiome initiation of *A. bisporus*.

1.1.1. STUDY RATIONALE

Few papers have been published which report on studies concerned with the involvement of bacteria in mushroom fruiting; those referred to above constitute the majority published since Eger first reported her findings in 1961. The scarcity of knowledge concerning the interaction between *P. putida* and *A. bisporus* and the apparent complexity of the process necessitated a broad based study. It seemed most appropriate to explore the interaction from a range of different perspectives, thereby gaining an overall view of the effect of *P. putida* on *A. bisporus*. The work presented in this thesis falls into four broad areas, each of which is concerned with a particular aspect of the interaction between bacterium and fungus.

Previous work found that basidiome stimulatory isolates of *P. putida* lost the ability to promote fruiting after long term storage. This suggested that the rough colonial forms, which arise from the smooth forms after several days growth on agar media, may be unable to promote

mushroom fruiting. A study of colony morphogenesis was undertaken to determine the significance of this phenomenon in terms of basidiome initiation. The production of rough colonial forms after several days growth suggested that these forms may be more stress-tolerant and the possibility that they represent long term survival forms was explored. In addition the taxonomic implications of the colony variants were considered.

The ecology of the interaction between *P. putida* and *A. bisporus* is not well understood. Many soil bacteria are chemotactic and can detect nutrient sources and migrate toward them. Chemotaxis confers upon bacteria a survival advantage and also facilitates adsorption to surfaces which is frequently necessary before a bacterium can exert a physiological effect on its host. The attraction and adhesion of *P. putida* to mushroom mycelium was investigated and the relationship between bacterium and fungus examined to determine whether there was evidence for a specific association, such as is found in the *Rhizobium*-legume, and *Agrobacterium*-plant interactions.

Two problems in particular have hindered studies investigating the involvement of *P. putida* in mushroom fruiting. The first arises from poor growth of *A. bisporus* on routinely used laboratory media and the second results from the extreme reluctance of the fungus to initiate basidiomata *in vitro*. Solutions were required to these problems before attempting to examine the mechanism by which the bacterium promotes fruiting. The development of a suitable bioassay for determining the effect of bacteria on basidiome initiation, would, in addition to facilitating genetical studies, also enable experiments on the nature of fruiting stimulus to take place.

Investigations into the bacterial genes responsible for promoting fruit body initiation were initiated after the problems discussed above were resolved, and after finding unequivocal evidence for the involvement of *P. putida* in mushroom fruiting. Genetic analysis relies on the ability to generate mutants and the availability of gene transfer systems. Methods for generating mutants were explored and the RP4::mini-Mu *in vivo* gene cloning and mapping system was developed in *P. putida*; a bacterium not well known from a genetical perspective.

CHAPTER TWO

FLUORESCENT PSEUDOMONADS ASSOCIATED WITH *AGARICUS BISPORUS*

2.1. INTRODUCTION

The pseudomonads are a large, diverse group of bacterla commonly found In soll, water, on diseased plants, and on spoiled foods (Palleroni 1984). By definition, pseudomonads are rod shaped, Gram negative, non-sporulating, polarly flagellated bacteria, which do not produce sheaths, or prosthecae. They are chemoorganotrophic with an aerobic and strictly respiratory type of metabolism which uses oxygen as the terminal electron acceptor, although in some cases nitrate can be used as an alternative electron acceptor. Some species are facultative chemolithotrophs, able to use hydrogen or carbon monoxide as energy sources. (Palleroni 1984).

The genus *Pseudomonas* has considerable sclentific and practical importance. Pseudomonads are among the most active participants in the process of mineralisation of organic matter in nature (Palleroni 1975). They are nutritionally versatile; some can utilize more than 100 substrates (Palleroni 1986), and others possess the ability to degrade recalcitrant compounds including, camphor (Rheinwald *et al.* 1973), toluene (Duggleby *et al.* 1977), 2,4,dichlorophenoxyacetic acid (Don & Pemberton 1981), 2,4,5-T (Kilbane *et al.* 1982) and penicillin (Beckman & Lessie 1981). Some members, for example *P. syringae* van Hall, and *P. viridiflava* (Burkholder) Dowson, cause disease of plants, others, including *P. aeruginosa* Schroeter (Migula) and *P. pseudomallei* (Whitmore) Haynes, cause disease of animals, while *P. cepacia* Burkholder (Palleroni & Holmes) can cause disease of both. Several pseudomonads, including *P. tolaasii* Paine and *'P. gingeri'* Preece & Wong, cause disease of cultivated mushrooms, especially *A. bisporus*, and particular strains of *P. putida* Trevisan (Migula) and *P. fluorescens* Trevisan (Migula) , are able to promote plant growth (Kloepper *et al.* 1980, Bakker *et al.* 1987). Several species, including *P. aeruginosa*, *P. fluorescens* and *P. putida*, are able to produce bacteriocins (Holloway & Krishnapillai 1975), toxins (Liu, 1974, Nair & Fahy 1973) and antibiotics (Howell & Stipanovic 1980, Salcher & Lingens 1980, Brisbane *et al.* 1987, Brisbane & Rovira 1988, Thomashow & Weller 1988).

2.1.1. THE FLUORESCENT *PSEUDOMONAS* GROUP

The genus *Pseudomonas* is divided into five groups based on the level of rRNA homology. The best known species of the genus belong to RNA group I which includes the type species *P. aeruginosa* and other saprophytic and phytopathogenic fluorescent pseudomonads, the non-pigmented denitrifying species of the 'stutzeri group' (Palleroni *et al.* 1970), and the non-pigmented strains of the 'alcaligenes group' (Ralston-Barrett *et al.* 1976).

Species belonging to rRNA homology group I which produce water soluble, fluorescent siderophore pigments (see below), are known as the fluorescent pseudomonads. *P. aeruginosa* and the phytopathogenic species; *P. syringae*, *P. viridiflava* and *P. cichorii* (Swingle) Stapp, form well defined categories, but the *P. putida/P. fluorescens* group is particularly heterogeneous and complex (Palleroni 1984).

Siderophores and iron acquisition

Iron is an essential element for growth of all living organisms and is relatively abundant in nature, however, in aerobic environments iron exists in the ferric form which is extremely insoluble (at physiological pH the solubility of ferric iron is ca. 10^{-17} M) rendering aquisition by micro-organisms (and higher organisms) difficult (Neilands 1984). Under conditions of iron limitation, almost all micro-organisms secrete low molecular weight, virtually ferric-iron (Fe(III)) specific ligands known as siderophores. These dissolve ferric iron, via complexation, from insoluble ferric oxyhydroxide polymers and naturally occurring chelating agents, and deliver iron to the cell, via high affinity transport systems (Neilands 1984).

Siderophores produced by pseudomonads of the *P. putida/P. fluorescens* group, known as pyoverdins (Meyer & Abdallah 1978) or pseudobactins (Teintze *et al.* 1981, Marugg *et al.* 1988), have a common structure consisting of a specific oligopeptide linked to a fluorescent chromophore, a quinoline derivative. These compounds have high affinities for iron (the binding constant for ferric iron at pH 7.0 is ca. 10^{25} (Cody & Gross 1987, Marugg *et al.* 1988)) and pseudomonads producing these compounds have been shown to possess a survival advantage in environments where iron is limiting (Kloepper *et al.* 1980, Bakker *et al.* 1987).

2.1.2. PSEUDOMONADS ASSOCIATED WITH *A. BISPORUS*

Pseudomonads form the dominant group of bacteria within the mushroom casing layer, representing more than half of the total bacterial population (Cresswell & Hayes 1979, Samson *et al.* 1987, Doores *et al.* 1987). Samson *et al.* (1987) found that the dominant species in the casing layer was *P. putida*, which comprised 47 % of the isolated bacteria.

Fluorescent pseudomonads, particularly those of the *P. putida/P. fluorescens* complex, are of considerable importance in mushroom cultivation. Several species are the cause of disease: *P. tolaasii* and *'P. gingeri'* are the cause of brown blotch (Paine 1919) and ginger blotch (Wong *et al.* 1982) respectively; *P. agarici* Young causes drippy gill disease (Young 1970) and an unidentified pseudomonad is associated with mummy disease (Schisler *et al.* 1968). Recently, a new disease of *A. bisporus*, caused by a pseudomonad resembling *P. agarici*, was described by Rainey & Cole (1988).

Pathogenicity of *P. tolaasii* has been shown to be directly related to the production of a diffusible toxin (Nair & Fahy 1973, Malcolm 1981, Peng 1986), but in most cases the disease causing factors have not been determined. Some pseudomonads, in particular *P. putida*, have been shown to play an important role in basidiome initiation (Hayes *et al.* 1969, Rainey & Cole 1987, see Chapter 4.0.) and pseudomonads antagonistic to *P. tolaasii* are also used as biological control agents (Fahy *et al.* 1981).

Olivier *et al.* (1978), Zarkower *et al.* (1984) and Doores *et al.* (1987) reported the occurrence of many saprophytic pseudomonads on mushroom caps, some of which were capable of producing a 'white line reaction' with *P. tolaasii* (Wong & Preece 1979, Peng 1986). These organisms were given the name *'P. reactans'* by Wong & Preece (1979), but Goor *et al.* (1986) found that 'white line reacting' organisms formed a heterogeneous group and therefore were unable to draw any taxonomic conclusions. Peng (1986) demonstrated that the white precipitate formed in agar was the result of a reaction between a compound, possibly an amide, produced by *'P. reactans'* and the toxin produced by *P. tolaasii*.

2.1.3. COLONY MORPHOGENESIS IN *PSEUDOMONAS*

Fluorescent *Pseudomonas* colonies growing on solid media frequently sector after several days growth. Colonies which arise from the cells of the sectored area differ morphologically (and sometimes physiologically) from the parent colony form (Shapiro 1986). Frequently this phase change is referred to as smooth/rough transition and has been reported in a range of bacteria, including *Xanthomonas phaseoli* (Corey & Starr 1957), *Salmonella typhimurium* (Lederberg & Iino 1956), *Neisseria gonorrhoeae* (Sparling *et al.* 1986) and *Rhizobium phaseoli* (Flores *et al.* 1988).

Rough forms usually possess lipopolysaccharide wall layers which differ both quantitatively and qualitatively from the lipopolysaccharide layers of smooth forms (Lucas & Grogan 1969, Jarrell & Kropinski 1977, Gerwe *et al.* 1987). As a consequence, a range of physiological properties are often affected, including resistance to antimicrobial compounds and bacteriophages (Nikaido & Hancock 1986), ability to cause disease (see Jarrell & Kropinski 1977) and ability to adhere to surfaces (Silverman *et al.* 1984, see Chapter 3.0.).

In some instances, for example, in *P. tolaasii* (Olivier *et al.* 1978), '*P. gingeri*' (Cutri *et al.* 1984) and *P. syringae* pv. *phaseolicola* (Gerwe *et al.* 1987), the smooth to rough transformation is accompanied by loss of virulence, but frequently there is no apparent physiological change and the rough forms can be easily overlooked. Unlike *S. typhimurium* and *N. gonorrhoeae*, phase variation in most pseudomonads is from smooth to rough and rarely occurs in reverse (Cutri *et al.* 1984). The mechanism by which smooth to rough variation occurs is not known (Govan *et al.* 1979), but it has been suggested that spontaneous mutation, phase variation, or loss of extra-chromosomal DNA may be responsible. Rangnekar (1988) reported that variation in the ability of a *Pseudomonas* sp. to utilize *meta*-chlorobenzoate was associated with tandem amplification and deamplification of DNA.

Smooth to rough reversion creates problems for workers who are concerned with aspects of the biology of smooth, originally isolated forms and survival of the desired colony type requires, at the very least, constant selection and subculturing. Govan (1975) reported that the mucoid form of *P. aeruginosa* was lost after 15 subcultures, despite careful and deliberate propagation of mucoid colonies. Govan found that addition of surfactants to culture media

helped to stabilize mucus production by *P. aeruginosa*, but he was unable to explain the reason for this. Enhanced stability of mucoid *P. aeruginosa* in aerated cultures was described by Govan *et al.* (1979) and they suggested that this could be due to more thorough dispersal of exopolysaccharide, resulting in improved oxygen and nutrient uptake. Govan *et al.* (1979) also found that in stationary broth culture the rough form possessed a growth rate advantage over the mucoid form.

The significance of rough colonial forms is not understood and their apparent inability to revert back to their previous smooth (and in some cases virulent) form is difficult to explain. Shapiro (1986) suggested that the phenomenon of colony morphogenesis was an expression of basic functions for cellular differentiation and for the organization of large populations. He stated that 'the ability to form organized multicellular aggregates is important in the establishment, survival, proliferation and spread of *Pseudomonas* populations in the wild'. The production of variant *Pseudomonas* colony forms in the wild has yet to be examined.

The significance of rough colonial forms of *P. putida*, in terms of their ability to trigger basidiome initiation, has not been determined, but comments by Eger (1972) and Vischer (1979) indicate that old stored cultures of *P. putida* are less effective at promoting fruiting of *A. bisporus*, than bacteria recently isolated from the casing layer. This was also indicated by Wood (1976) who was unable to achieve fruiting using the old stored isolates of Hume & Hayes (1972).

Cutri *et al.* (1984) noted that the rough forms of *P. tolaasii* and '*P. gingeri*' differed from the smooth forms with respect to several morphological and biochemical characteristics. Changes in the characteristics of other pseudomonads showing a smooth to rough reversion have also been reported (Lucas & Grogan 1969, Govan 1975, Gerwe *et al.* 1987). It is possible that in some instances the differences between forms may be greater than the difference between species, or biotypes, and this has implications for pseudomonad taxonomy which require consideration.

2.1.4. STUDY AIMS

This chapter reports on the characterization and identification of *Pseudomonas* isolates used in subsequent studies. In addition, the phenomenon of colony morphogenesis is examined and the

morphogenetical capabilities of a single *P. putida* and *P. tolaasii* colony are investigated. The taxonomic implications of the different colonial forms are also considered.

The production of rough colonial variants *in vivo* is examined and the ability of rough colonial forms of a basidiome stimulatory *P. putida* isolate to promote mushroom fruit body initiation is determined.

The production of rough colonial variants on rich media after several days growth, suggested that these forms may be stress tolerant and better adapted to withstand adverse conditions. The possibility that they may represent a long term survival form is investigated.

2.2. MATERIALS AND METHODS

2.2.1. ISOLATION AND IDENTIFICATION OF BACTERIA

Isolation of bacteria

Samples of casing material (1 g) were removed from the casing layer of commercial mushroom farms, suspended in 99 ml sterile distilled water and shaken for 30 min at room temperature. Appropriate dilutions were made in phosphate buffered saline (PBS) and 0.1 ml was spread on Petri dishes containing 25 ml King's medium B (KB) (King *et al.* 1954). The plates were incubated at 28 °C, for 48 h and colonies producing a green/yellow fluorescent pigment were checked for purity by streaking on fresh KB plates. Pseudomonads were also isolated directly from mushroom caps with an inoculating loop and streaked on KB plates.

Storage of cultures

Single colony isolates were grown overnight in 5 ml of nutrient broth and 0.8 ml placed in a sterile 1.5 ml Eppendorf tube containing 0.2 ml sterile glycerol. The contents were mixed vigourously, the tubes labelled and stored at -80 °C. Culture characteristics were entered into a data base computer software package, Dbase III (Ashton-Tate). Isolates were prescribed a three digit number preceded by PMS (Plant & Microbial Sciences). Where it was necessary to distinguish between smooth and rough colony variants a letter was placed after the isolate number. For example, PMS118S refers to the smooth colonial form of PMS118, similarly, PMS118R refers to the rough form and PMS118Rr refers to the rough form produced by the first rough colonial variant.

Morphological and biochemical tests

Several hundred fluorescent isolates were collected from a range of mushroom environments, purified and stored at -80 °C, but only those strains used in the work described in this thesis were characterized fully. Two non-fluorescent mutants, derived independently from *P. putida* PMS118R, PR225 and PR226, were also examined from a taxonomic perspective. PR225 arose

spontaneously and PR226 was isolated after treatment of PMS118R with UV light (see section 5.2.2.).

All plates and tests were performed at 28 °C (*P. aeruginosa* was incubated at 37 °C) unless otherwise stated. Pigmentation and colony morphology were recorded after 48 h growth on KB and King's medium A (KA) (King *et al.* 1954). Cellular morphology and motility were determined by examining cells (less than 24 h) grown in an infusion of macerated mushroom tissue (Young 1970) by light microscopy. Flagella were detected using Ryu's stain (Kodaka *et al.* 1982). Tests for catalase, oxidase, oxidation-fermentation (O-F), arginine dihydrolase and denitrification were conducted according to King & Phillips (1985). Growth at 4 °C and 42 °C, gelatin hydrolysis and white line tests were performed as described by Zakower *et al.* (1984). Determination of Poly-β-hydroxybutyrate (PHB) inclusions, Gram reaction, KOH solubility, levan and hydrogen sulphide production were performed as outlined by Fahy & Hayward (1983). The effect of isolates on basidiome initiation of *A. bisporus* and mycelial growth was determined by the methods outlined in Chapter 4.0. (see section 4.2.3.).

API 50CH tests were used to determine the ability of selected isolates (PMS117S, PMS117R, PMS118S, PMS118R, PMS118Rr, PMS195, PMS196, PMS234, PMS382, PR225, PR226) to assimilate a range of carbon sources. The basal medium for these tests was prepared as described by Goor *et al.* (1986). The final concentration of the substrates was 0.2 % and growth was observed after 1,2,4 and 7 d.

Some isolates and their various colony forms were identified by gas chromatography of whole cell fatty acids using the Hewlett Packard 5898A Microbial Identification System. This was located at the Ministry of Agriculture Fisheries and Food, Harpenden Laboratory, Harpenden, U.K. The procedure used was that outlined in Hewlett Packard 'Application Note 228-41' by Miller & Berger (1985) and involved growing bacteria on trypticase soy broth agar (TSBA) for 24 h at 28 °C, suspending the cells in screw capped vials where they were saponified to release fatty acids, methylated, extracted with hexane and ethyl ether and washed in NaOH before automated analysis and identification. Identification involved a comparison between the fatty acid profile of the sample and a library of fatty acid profiles contained within the computer memory.

2.2.2. COLONY MORPHOGENESIS

The lineages of a single, smooth (originally isolated) colony, of *P. putida* PMS118S and *P. tolaasii* PMS117S, grown under conditions promoting maximum colony diversity, were followed.

A single smooth colony of each species was inoculated into 5 ml of *Pseudomonas* agar F (PAF) broth contained in McCartney bottles and incubated at 28 °C without shaking. After 1 wk, the McCartney bottles were vortex-mixed to break up bacterial aggregates and cells were removed with an inoculating loop, streaked onto PAF and incubated at 28 °C for 3 d. Morphologically distinct, single colonies, were selected from the plates and re-inoculated into PAF broth and the procedure repeated. The lines of descent were traced by a simple book-keeping procedure as described by Shapiro (1986) and colonies photographed under a compound microscope using oblique illumination.

The toxin producing ability of *P. tolaasii* subclones was examined using the white line reaction (Preece & Wong 1979). A single streak of '*P. reactans*' was place in the centre of a plate of PAF and single colonies of *P. tolaasii* streaked perpendicular to this line. The presence of a 'white line' was determined after 3 d incubation at 28 °C.

2.2.3. PRODUCTION OF ROUGH COLONIAL FORMS IN PEAT CASING SOIL

The moisture content of a commercial peat casing material was adjusted to field capacity and small volumes (5 - 10 g) were placed in 100 ml flasks and sterilized by autoclaving (25 min). Sterility was checked by suspending 0.1 g in 9.9 ml of PBS and spreading 0.1 ml aliquots onto nutrient agar plates. The flasks containing casing material were inoculated with 5 ml of a suspension of *P. putida* PMS118S or *P. tolaasii* PMS117S, 10^6 cfu ml^{-1}, in sterile distilled water. The flasks were incubated at 25 °C and after 3 wk, 0.1 g of casing material was suspended in 9.9 ml sterile PBS, shaken for 30 min at room temperature and then diluted in PBS before spreading 0.1 ml aliquots onto fresh plates of KB. Plates were incubated at 28 °C for 3 d and then examined for the presence of rough colonial forms.

2.2.4. EXAMINATION OF THE ABILITY OF ROUGH AND SMOOTH COLONIAL FORMS TO WITHSTAND STRESS

Two rough colonial forms of *P. putida* PMS118S are commonly produced on agar media. *P. putida* PMS118S sectors after 3 - 4 d producing *P. putida* PMS118R which sectors producing a further rough form, *P. putida* PMS118Rr. Usually only a single rough colony form is produced by the smooth form of *P. tolaasii* PMS117S on agar media, PMS117R. The possibility that the rough colonial forms represent stress-tolerant forms was explored.

Growth under conditions of nutrient limitation.

P. putida PMS118 and *P. tolaasii* PMS117 colonial forms were grown overnight in an orbital incubator at 28 °C, in 100 ml flasks, containing 10 ml of KB broth. Fresh inoculum from these cultures was used to seed 250 ml flasks containing 50 ml of 1/100 strength, and full strength, KB broth with 10^3 - 10^4 cfu ml^{-1}. Growth of each colonial form was monitored by removing samples at regular intervals and determining the number of colony forming units per ml by spreading cells on fresh plates of KB with a spiral plating device. The plates were incubated for 48 h, at 28 °C, before counting colonies. Experiments were duplicated.

Resistance to UV light

Resistance of *P. putida* PMS118 and *P. tolaasii* PMS117 colonial forms to UV light was determined using the method of Kloepper & Schroth (1981). Bacteria were grown overnight in 5 ml Luria (L) broth (Miller 1972), washed in 0.01 M $MgSO_4.7H_2O$ and suspended in 5 ml of 0.1 M $MgSO_4.7H_2O$ to a density of 10^8 cfu ml^{-1}. The suspension was poured into a glass Petri dish and placed (with lid removed) 35 cm below a UV light (254 nm) source. Samples (0.1 ml) were removed from the dish every 10 s, for 50 s and the number of bacteria surviving at each time interval determined using a spiral plating device. The plates were incubated for 48 h, at 28 °C, before counting colonies.

Production of siderophores

The possibility that rough colonial forms produce greater amounts of siderophores than the smooth forms was investigated. In addition, the siderophore compounds produced by *P. putida* PMS118 and *P. tolaasii* PMS117 were examined.

Siderophore production was determined by growing each *P. putida* PMS118 and *P. tolaasii* PMS117 colonial form in 100 ml flasks containing 20 ml succinate broth, in an orbital incubator, at 28 °C (Meyer & Abdallah 1978). The cells were removed from the broth by centrifugation after 48 h growth, the pH adjusted to pH 7.0 with 1N HCl and the supernatant sterilized by filtration through a 0.22 μm Millipore filter. The absorption spectra of the supernatant was determined using a Perkin Elmer 554 scanning UV-VIS spectrophotometer. The absorbance of the Fe(III)-siderophore complex was measured after addition of 500 μM $FeCl_3$ to the supernatant.

Respiration

To determine whether *P. putida* PMS118 and *P. tolaasii* PMS117 colonial forms possess different rates of respiration, carbon dioxide evolution was measured over a 24 h period. Capped test tubes (15 ml) equipped with a rubber septum were filled with 5 ml of succinate broth (Meyer & Abdallah 1978) and inoculated with 10^6 cfu ml^{-1} from overnight cultures in the same broth. Carbon dioxide evolution was determined using a Shimadzu gas chromatograph (GC8A) equipped with an Alltech CTR1 column coupled to a Shimadzu C-R3A Chromatopac. The gas chromatograph was calibrated with a standard gas mix obtained from Phase Separation (Queensferry, Clwyd, U.K.). In addition to measuring carbon dioxide evolution during growth of cells in succinate broth (an iron limited medium), carbon dioxide evolution was also measured during growth of cells in succinate broth supplemented with 100 μM $FeCl_3$. Treatments were duplicated.

Motility

The swarming abilities of different colonial forms of *P. putida* PMS118 and *P. tolaasii* PMS117 was determined by examining the diameter of colonies on plates of semi-solid nutrient agar after growth.

An inoculating needle was dipped into an overnight culture in nutrient broth and then touched onto the surface of 1/10 strength, semi-solid (0.3 % agar), nutrient agar plates. These were then incubated at 28 °C and colony diameter measured after 24 h incubation.

2.3. RESULTS AND DISCUSSION

2.3.1. CHARACTERIZATION OF *PSEUDOMONAS* ISOLATES

Morphological and biochemical characteristics

All isolates produced 3 - 4 mm diam domed, smooth; opaque, cream or translucent colonies with entire margins, after 48 h growth on KB. A yellow/green water soluble pigment which fluoresced under UV (254 nm) light was produced by all isolates, except the non-fluorescent mutants PR225 and PR226, after growth on KB. With the exception of *P. aeruginosa*, no isolate produced a fluorescent pigment on KA. PMS382 produced, in addition to a yellow/green fluorescent pigment on KB, an orange, water soluble, non-fluorescent pigment on KB, KA, and nutrient agar. All isolates were Gram negative, catalase and oxidase positive and with the exception of *P. aeruginosa*, motile by two or more polar flagella; *P. aeruginosa* possessed only a single polar flagellum. Glucose was metabolized by all isolates, but only in the presence of oxygen and PHB inclusions were not detected. No isolate produced levan from sucrose and only *P. aeruginosa* grew at 42 °C. All isolates, with the exception of *P. aeruginosa*, grew at 4 °C. Table 2.1 shows the dissimilar characters of the bacterial isolates.

On the basis of biochemical characteristics, the isolates were considered members of rRNA group I of the genus *Pseudomonas* (Palleroni 1984). The key presented in *Bergey's Manual of Determinative Bacteriology* (Palleroni 1984) was used to identify the isolates to species level. Arginine dihydrolase positive, oxidase positive organisms were placed within the *P. fluorescens* complex encompassing *P. aeruginosa*, *P. fluorescens* and *P. putida*. *P. fluorescens* isolates were distinguished from *P. aeruginosa* on the basis of multitrichous flagellation, lower maximum growth temperature and absence of pyocanin production on KA, and from *P. putida* on the basis of gelatin hydrolase activity. One isolate, PMS234, failed to hydrolyse gelatin, but was able to denitrify, and thus appears intermediate between *P. putida* and *P. fluorescens*. *P. tolaasii* was distinguished from *P. fluorescens* on the basis of the 'white line in agar' test. Two *P. fluorescens* isolates, PMS273 and PMS274, produced a 'white line in agar'

Table 2.1. Dissimilar characteristics of *Pseudomonas* Isolates

Reference cultures		Arginine	Gelatin	H_2S	NO_3- N_2	W.L.tol[1]	W.L.rea[2]	Basidiocarp[3] initiation, mycelial growth
P. aeruginosa (OT11)		+	+	-	+	-	-	-,-
P. agarici (PDDCC 2656)		+	-	+	-	-	-	-,-
P. fluorescens (ATCC 13525)		+	+	-	-	-	-	NT
P. putida (ATCC 12633)		+	-	-	-	-	-	NT
P. reactans (NCPPB 3149)		+	+	-	-	+	-	-,--
P. tolaasii (PDDCC 4227)		+	+	-	-	-	+	-,--
Isolate number	**Identity**							
PMS114	P. tol[4]	+	+	-	-	-	+	-,--
PMS115	P. tol	+	+	-	-	-	+	-,--
PMS117S	P. tol	+	+	-	-	-	+	-,--
PMS117R	P. tol	+	+	-	-	-	-	-/+,+
PMS118S	P. put	+	-	-	-	-	-	+,+
PMS118R	P. put	+	-	-	-	-	-	+,+
PMS118Rr	P. put	+	-	-	-	-	-	+,+
PR225	P. put	+	-	-	-	-	-	-,-
PR226	P. put	+	-	-	-	-	-	-,-
PMS126	P. ag?	-	-	+	-	-	-	-,-
PMS132	P. put	+	-	-	-	-	-	+,+
PMS157	P. flu	+	+	-	-	-	-	-,0
PMS195	P. put	+	-	-	-	-	-	+,+
PMS196	P. put	+	-	-	-	-	-	+,+
PMS220	P. put	+	-	-	-	-	-	+,+
PMS233	P. put	+	-	-	-	-	-	+,+
PMS234	P. put/flu	+	-	-	+	-	-	+,+
PMS273	P. flu	+	+	-	-	+	-	-,--
PMS274	P. flu	+	+	-	-	+	-	-,-
PMS382	P. flu	+	+	-	-	-	-	-,--

[1]White line in agar when plated against *P. tolaasii*

[2]White line in agar when plated against '*P. reactans*'

[3]Effect on basidiome initiation and mycelial growth. Symbol to left of comma; + = promotes basidiome initiation, - = does not promote basidiome initiation; -/+, primordia rarely produced. Symbol to right of comma; + = stimulates mycelial growth, 0 = no effect, - = inhibits, -- = inhibits growth at a distance > 1 mm from bacterial colony.

[4]P. tol = *P. tolaasii*, P. put = *P. putida*, P. ag = *P. agarici*, P. flu = *P. fluorescens*, P. put/flu = intermediate between *P. putida* and *P. fluorescens*, P. flu(WL) = *P. fluorescens* with ability to form white line in agar when plated alongside *P. tolaasii*.

when plated alongside *P. tolaasii*, typical of 'white line reacting organisms' (Goor *et al.* 1986). PMS126 was similar to *P. agarici* and failed to produce arginine dihydrolase, was gelatin hydrolase negative, oxidase positive and produced hydrogen sulphide. These characteristics support its placement within the *P. agarici/P. cichorii* group, but further work its required to confirm its identity. The two mutants of *P. putida* PMS118R, PR225 and PR226, displayed identical biochemical characteristics to PMS118R,

Nutritional characteristics

The following carbohydrate compounds were used by all isolates; glycerol, ribose, D-glucose, D-fructose, gluconate and 2 keto-gluconate. None utilized D-arabinose, L-xylose, β methyl-xyloside, L-sorbose, rhamnose, dulcitol, methyl α mannoside, methyl α D-glucoside, amygdaline, arbutin, salicin, cellobiose, maltose, aesculin, lactose, D-melibiose, inulin, D-melezitose, D-raffinose, starch, glycogen, α gentiobiose, D-turanose, D-tagatose, D-fucose and L-fucose. Dissimilar utilization of other carbohydrate compounds is shown in Table 2.2.

Table 2.2. Dissimilar carbon source utilization patterns of *Pseudomonas* isolates.

	Isolate number						
Carbon source	PMS117S PMS117R	PMS118S PMS118R PMS118Rr	PMS195	PMS196	PMS234	PMS382	PMS225 PMS226
Erythritol	+	-	-	-	-	-	+
L-arabinose	-	-	+	+	+	+	+
D-xylose	-	-	-	-	+	-	+
Adonitol	+	-	-	-	-	-	+
D-galactose	+	-	+	-	+	+	+
D-mannose	+	-	+	+	+	+	+
Inositol	+	-	-	-	-	+	+
Mannitol	+	-	+	-	+	+	+
Sorbitol	+	-	-	-	-	-	+
N-acetyl glucosamine	+	-	-	-	+	+	+
Sucrose	-	-	-	-	-	+	+
Trehalose	-	-	-	-	+	+	+
Xylitol	+	-	-	-	-	-	+
D-lyxose	+	-	-	-	-	-	-
D-arabitol	+	-	+	-	+	+	+
L-arabitol	+	-	-	-	-	-	+
5 keto-gluconate	+	-	-	-	-	+	-

The nutritional characteristics of the isolates confirmed the identifications made on the basis of biochemical characteristics. The pattern of carbohydrate utilization by PMS117 (smooth and rough colonial forms) was identical to that described by Goor *et al.* (1986) for *P. tolaasii* isolates, with the exception of trehalose, which was not used by PMS117. Fahy (1981), however, reported that trehalose utilization by *P. tolaasii* was variable and Young (1970) found that the two isolates he examined failed to produce acid from trehalose.

The three isolates identified on the basis of biochemical characteristics as *P. putida*; PMS118, PMS195 and PMS196 and the isolate intermediate between *P. putida* and *P. fluorescens*, PMS234, displayed variation in their ability to utilize carbon sources. None of these isolates were able to utilize erythritol, adonitol or *meso* inositol and they varied in their ability to utilize L-arabinose, D-xylose, D-galactose, D-mannose and mannitol, but these characteristics are in agreement with those presented by Palleroni (1984) for *P. putida* isolates. Utilization of trehalose by PMS234 is an unusual characteristic of *P. putida*, but Barrett *et al.* (1986) found that 6 % of *P. putida*, biotype B isolates where able to utilize this compound. On the basis of the nutritional characteristics, PMS195 most likely belongs to biotype B, while PMS118 and PMS196 probably belong to biotype A. Despite the ability to denitrify, PMS234 is more *P. putida* like than *P. fluorescens* like and probably also belongs to biotype B of *P. putida*

The nutritional characteristics of PMS382 are consistent with those of *P. fluorescens* (Palleroni 1984) and its inability to utilize either erythritol, or sorbitol, distinguishes it clearly from *P. tolaasii* and *P. gingeri* (Goor *et al.* 1986). With the exception of *meso*-inositol, *N*-acetyl-glucosamine, sucrose and 5-keto-gluconate utilization, and the inability to produce hydrogen sulphide from cysteine, PMS382 possessed nutritional and biochemical characteristics identical to those of 'mummy disease' causing isolates (Goor *et al.* 1986).

The ability to promote basidiome initiation and stimulate the rate of mycelial extension was confined to *P. putida* isolates. Isolates which failed to promote basidiome initiation, either caused inhibition of mycelial growth once contact of the mycelium with the bacterial colony was made, or inhibited mycelial growth at a distance. *P. tolaasii* strains and some *P. fluorescens* isolates, most notably PMS382, caused an inhibition zone of greater than 5 mm. This was most likely due to the

production of diffusible, anti-fungal compounds. Toxin production by *P. tolaasii* is well known (Nair & Fahy 1973, Malcolm 1981, Peng 1986) and was probably responsible for the observed inhibition by *P. tolaasii* isolates. It would be of interest to examine the anti-fungal metabolites produced by PMS382.

Smooth and rough colonial forms of *P. putida* PMS118 possessed identical biochemical and nutritional characteristics and all forms were able to promote basidiome initiation and stimulate the rate of mycelial growth. This indicates that the ability of *P. putida* PMS118S to promote basidiome initiation of *A. bisporus* is not lost during the transformation from smooth to rough. Nevertheless, the ability of rough colonial forms from a range of *P. putida* isolates to promote basidiome initiation was not examined and it is possible that in some strains, the rough forms may lack this ability. Caution should still be exercised when examining the effect of stored cultures, or old *P. putida* strains, on basidiome initiation.

The two colonial forms of *P. tolaasii* PMS117 also possessed identical nutritional and biochemical characteristics, with the exception of the 'white line in agar' test. The failure of the rough colonial form to cause disease, a result of its inability to produce a toxin, has been reported previously (Cutri *et al.* 1984). Interestingly, the rough colonial form promoted the rate of *A. bisporus* mycelial growth and was also able to promote basidiome initiation, although this ability was variable (Table 2.1). The smooth and rough colonial forms of *P. tolaasii* PMS117 also displayed a difference in the length of time required to hydrolysed gelatin; the smooth form required less than 24 h, whereas the rough form took up to 2 wk.

Stolp (1961) (as quoted by Lelliott *et al.* 1966) noted that a number of pathogenic pseudomonads were very closely related to the ubiquitously occurring saprophytic species, such as *P. putida* and *P. fluorescens*, and suggested that under certain selective conditions, pathogenic pseudomonads may arise, via mutation, from saprophytic species. It is possible that the rough non-pathogenic form of *P. tolaasii*, which lacks the ability to produce a toxin, represents the saprophytic form, from which the toxin producing, blotch causing organism arises. Lelliott *et al.* (1966) suggested that an exudate from mushroom caps may provide the selective pressure. The possible transformation between 'species' by members of the

P. putida/P. fluorescens complex raises many interesting questions which deserve further consideration.

The nutritional characteristics of the *P. putida* PMS118R mutants, PR225 and PR226, were identical but differed from the wild type (Table 2.2). Nevertheless, with the exception of erythritol utilization, the nutritional characteristics of the mutants are consistent with those of *P. putida* (Palleroni 1984, Barrett *et al.* 1986). This is an unusual result and suggests the possibility that the mutants may not have arisen from PMS118R. This, however, is extremely unlikely, especially as they were generated independently. Perhaps PMS118R synthesizes enzymes enabling metabolism of a range of carbon sources, but does not possess the full complement of enzymes. The mutant colonies were considerably more 'sticky' than those of the wild type which suggests that alterations within the cell wall material (lipopolysaccharide layer and membrane bound proteins) have occurred. The cell wall plays a major role in regulating the excretion of compounds (Nikaido & Hancock 1986) and the altered layer may enable a greater range of enzymes to be excreted. Alternatively, an operon coding for a range of carbohydrate degrading enzymes may have been activated. Cell wall permeability may also account for the varying time required by *P. tolaasii* isolates to hydrolyse gelatin:- In a preliminary experiment, a range of *P. tolaasii* isolates were examined for their ability to hydrolyse gelatin. Some isolates hydrolysed gelatin overnight, while others required several weeks before signs of liquification were evident. Depending on the method of determining the presence of gelatin hydrolase, some of these isolates could be classified as toxin producing isolates of *P. putida*.

Fatty acid analysis of whole bacterial cells

Cellular fatty acid composition of *Pseudomonas* isolates is shown in Table 2.3. The different colonial forms of *P. putida* PMS118 and *P. tolaasii* PMS117 displayed differences in their fatty acid profiles and were assigned different species identifications by the Hewlett Packard Microbial Identification System. Frequently the computer was unable to find a satisfactory match between the fatty acid profile of an isolate and the library profiles. This was indicated by percentage similarity values of less than 0.3 (Table 2.3).

Table 2.3. Cellular fatty acid composition of *Pseudomonas* isolates

Isolate	Straight chain acids											Hydroxy acids			Cyclo-propane acids		Match	% Sim
	10:0[a]	12:0	14:0	15:0	16:1 cis	16:1 trans	16:0	17:0	18:1 cis	18:1 trans	18:0	3-OH 10:0	2-OH 12:0	3-OH 12:0	17:0	19:0		
PMS118S	-[b]	1.4	0.4	0.5	18.0	-	30.9	0.35	16.6	-	0.9	2.7	5.7	4.0	18.3	0.4	P. tol[c]	0.267
PMS118S*	0.4	1.4	0.3	0.3	24.3	3.0	29.5	0.2	-	16.8	0.5	4.1	5.0	4.0	9.4	0.2	P. put	0.523
PMS118R	-	1.6	0.5	0.3	17.9	-	30.5	-	17.4	-	0.8	3.0	5.9	4.2	17.4	0.5	P. tol	0.295
PMS118R*	0.4	1.6	0.4	0.4	16.7	3.8	28.5	0.3	-	15.5	0.5	1.6	5.7	4.4	0.3	0.4	P. put	0.152
PMS118Rr	0.2	2.5	0.4	0.4	26.6	4.6	-	0.2	17.1	-	0.6	2.9	5.2	4.1	7.6	-	P. put	0.634
PMS118Rr*	-	2.5	0.6	0.3	23.2	5.3	31.5	-	14.8	-	0.7	3.5	5.4	4.2	8.1	-	P. put	0.534
PR225	-	1.8	0.4	-	32.2	-	28.6	0.3	16.5	-	0.9	2.4	5.3	4.1	6.9	0.7	P. tol	0.677
PR226	-	1.7	0.4	0.4	14.2	-	31.0	0.6	11.4	-	1.0	2.4	5.7	4.0	23.6	3.7	P. tol	0.059
PMS117S	-	1.3	0.4	0.5	13.5	-	33.1	0.6	-	12.0	1.2	2.3	5.8	4.0	23.1	2.3	P. tol	0.099
PMS117S*	0.15	1.6	0.4	0.5	17.3	-	32.8	0.5	13.7	-	1.0	2.8	5.1	4.2	17.9	1.7	P. tol	0.347
PMS117R	-	2.7	0.4	0.6	21.0	-	30.8	0.5	15.9	-	0.9	2.9	5.0	4.2	13.8	1.0	P. tol	0.604
PMS117R*	0.1	2.6	0.4	0.5	23.2	-	30.1	0.4	-	16.9	0.7	3.2	4.8	4.6	11.0	0.8	P. tol	0.733

[a]Number to left of colon refers to number of carbon atoms; number to right refers to number of double bonds; 2- and 3-OH refer to hydroxy acid.

[b]Number refers to percentage of total acids, -, not detected.

[c]P. tol = *P. tolaasii*, P. put = *P. putida*.

*Cultures not analysed within 24 h.

The differences between the fatty acid profiles of *P. putida* and *P. tolaasii* contained within the Microbial Identification System library are not marked; the main differences are in the amount of 17-cyclopropane acids (cy) - 4.5 % *P. putida*, 9.2 % *P. tolaasii*; 16:0 straight chain acids - 28 % *P. putida*, 31 % *P. tolaasii*, and 16:1, *cis* or *trans*, straight chain acids - 24 % *P. putida*, 29 % *P. tolaasii*. The amount of 16:0 remained constant between colonial forms, but the amount of 17 cy and 16:1 altered markedly depending on the colonial form. The rough forms of both species contained greater amounts of 16:1 than the smooth forms, but the smooth colonial forms contained more 17 cy than the rough forms. These differences between colonial forms were at least as great as the differences between species. Interestingly, the 'rougher' the colonial form, the better the match with the expected library profiles. This suggests that the fatty acid profiles contained within the computer identification system have arisen from isolates which were predominantly rough prior to analysis. A second set of isolates sent to Harpenden, were, unlike the first set, not analysed immediately. Consequently, when grown overnight in preparation for fatty acid extraction, a large proportion of rough colonial forms would have been mixed in with the smooth forms. The 'smooth' colonial forms of this batch of samples were found to match well with the expected library profiles (Table 2.3). This result highlights the need to distinguish carefully between colony forms if fatty acid analysis of whole bacterial cells is to be used successfully for identifying closely related species.

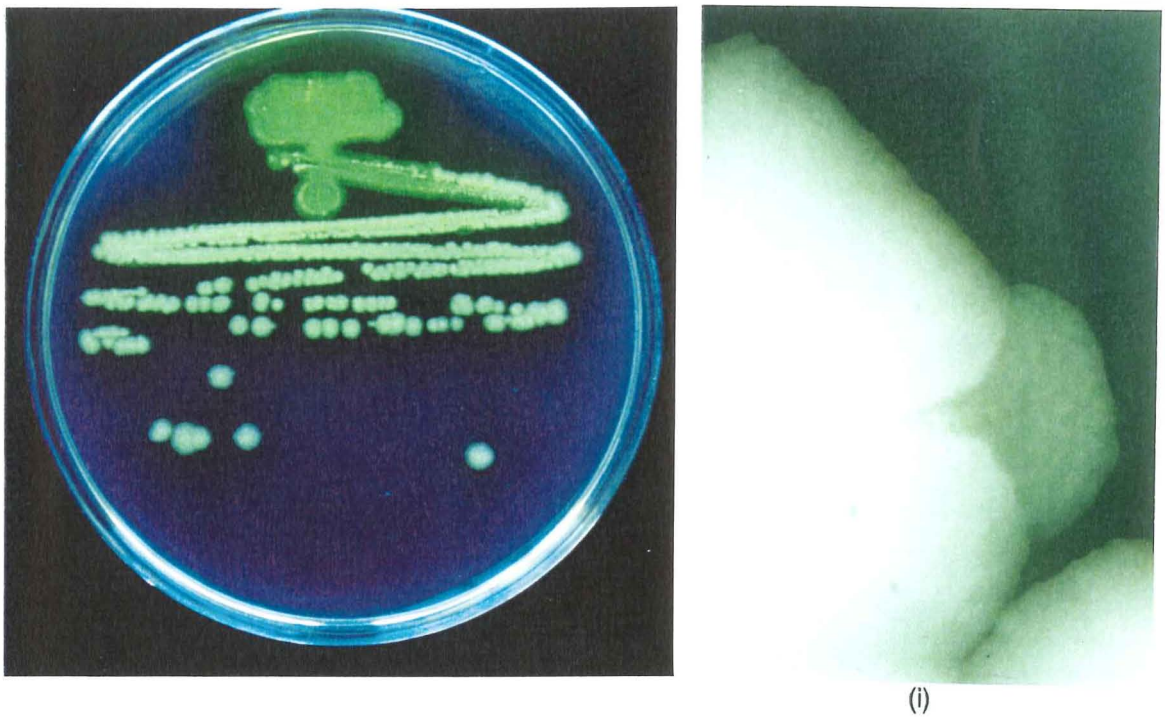
The two non-fluorescent *P. putida* mutants, PR225 and PR226, showed differences in their fatty acid profiles. The presence of 3.7 % 19 cy in PR226 resulted in an uncertain identification, but PR225 was identified with a good degree of accuracy as *P. tolaasii*. The taxonomy of mutant colonies clearly warrants further investigation.

2.3.2. COLONY MORPHOGENESIS

On solid KB media sectoring of smooth colonies was observed after several days growth (Figs 2.1 and 2.2). The smooth form could be maintained on solid media by selecting smooth colonies and subculturing weekly.

The lineages of a single *P. putida* PMS118S and *P. tolaasii* PMS117S colony are shown in Figs 2.3 and 2.4. Both species displayed considerable morphogenetical capabilities and a

Fig. 2.1. *P. putida* PMS118S on KB after 3 d growth. Note sectoring of colonies situated at the plate periphery and the production of the rough colonial variant, PMS118R.



(i)

Fig. 2.2. *P. tolaasii* PMS117S on KB after 3 d growth. White colonies are the smooth colonial type, translucent colonies are the rough colonial type. Smooth colonies have not arisen in the crowded region of the plate, probably due to their inability to compete with the rough colonial form (see text). Rough colonies have not grown amongst the smooth colonies, possibly because of the production of toxin production by the smooth form. (i) Sectoring of PMS117S and emergence of the rough colonial form, PMS117R.

Fig. 2.3. *P. putida* **lineage**

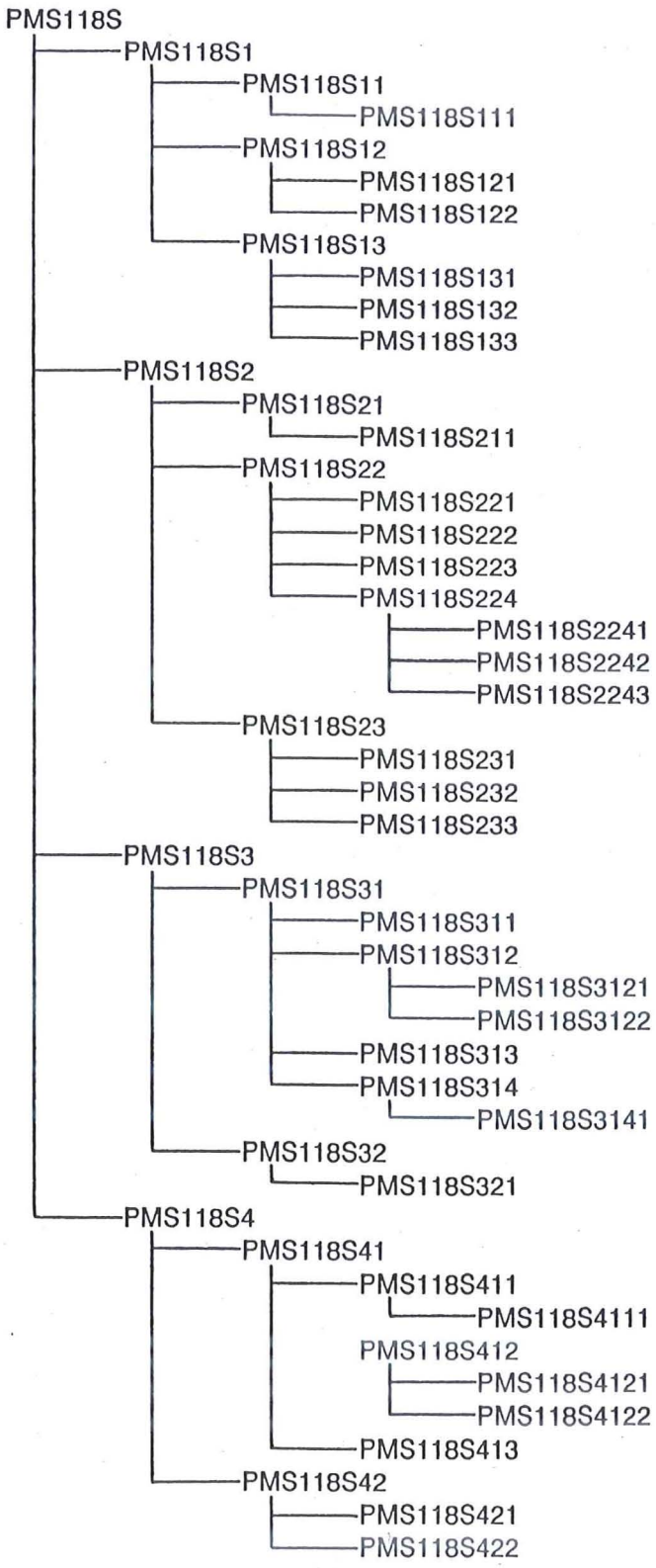
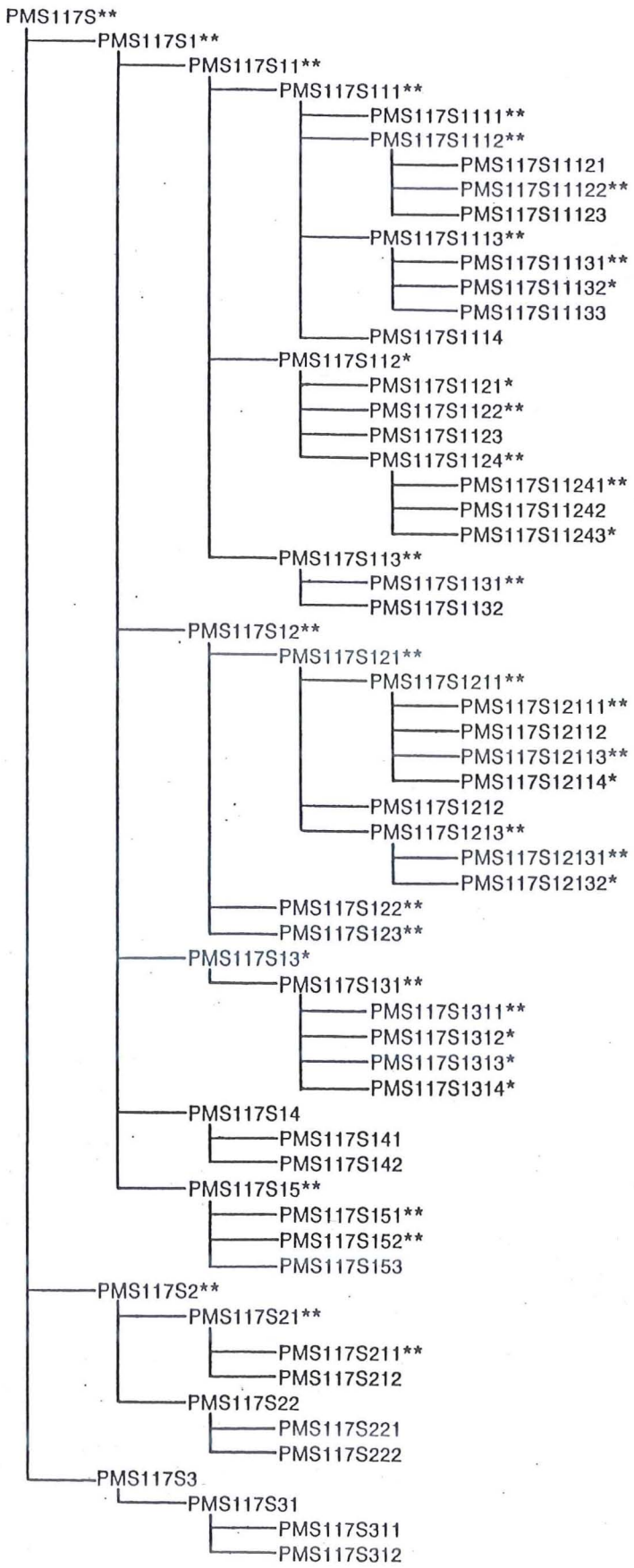


Fig. 2.4. *P. tolaasii* **lineage**



** = Production of toxin.

* = Weak production of toxin.

diverse range of colonial forms were produced (Figs 2.5 - 2.8). The inheritance of toxin producing ability by *P. tolaasii* PMS117 colonial forms was examined and is also shown in Fig. 2.4. Rough, non-toxin producing colonial forms never gave rise to toxin producing colonies, but weak toxin producing colonial variants gave rise to colonies which excreted a normal amount of toxin.

The apparent inability of rough forms to revert back to smooth forms is difficult to understand, especially in *P. tolaasii*, where the rough colonial form is unable to cause disease of mushroom caps. There seems little benefit to be gained by becoming permanently avirulent and therefore it is likely that under certain conditions (see above), the rough form may revert back to a pathogenic state. *P. aeruginosa* isolates associated with cystic fibrosis almost always occur in a mucoid form, which arises *in vivo* from a non-mucoid form as a result of a chromosomal mutation (see Govan & Harris 1986). Mucoid forms, like the smooth colonial form of *P. tolaasii* PMS117 or *P. putida* PMS118, are unstable *in vitro* and rapidly produce non-mucoid variants, however, Martin (1973) found mucoid forms were produced from non-mucoid forms *in vitro* after treatment of the non-mucoid form with a phage.

Loss of a plasmid was suggested by Cutri *et al.* (1984) to account for the inability of the rough form to revert to the smooth. Preliminary experiments which examined the plasmid profiles of a range of casing layer derived *Pseudomonas* isolates revealed the presence of a large (ca. 100 kb) plasmid in both smooth and rough colonial forms of *P. tolaasii* PMS117 (Appendix C). While this suggests that colony transformation is not associated with loss of a plasmid, it does not exclude the possibility that loss of a very large undetected plasmid may result in this transformation. Such a plasmid could be regained by conjugation from suitable donors.

No pattern of hereditary transmission of colony morphology was detected amongst the subclones, but an inherent control mechanism did appear to exist within the cells of each subclone. This was concluded after separate growth of multiple inocula from a single colony, produced the same number and morphological colony types when plated on agar plates. Similar

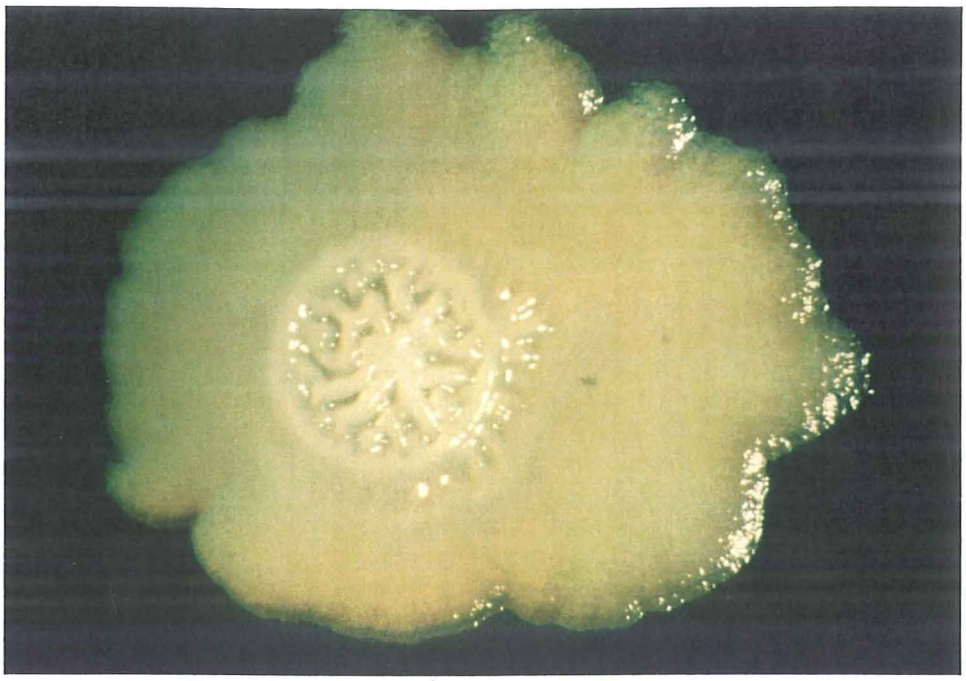


Fig. 2.5. A single *P. putida* PMS118 colonial form, PMS118S4122 (see Fig. 2.3), produced by PMS118S after growth in stationary broth culture.

Fig. 2.6. A single *P. putida* PMS118 colonial form, PMS118S422 (see Fig. 2.3), produced by PMS118S after growth in stationary broth culture.

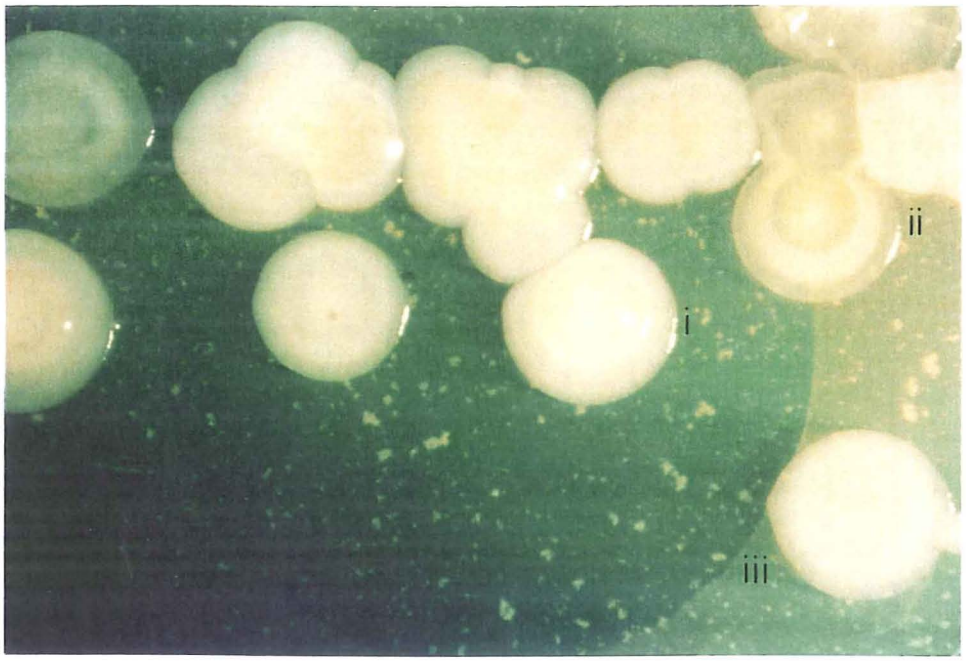


Fig. 2.7. A range of *P. tolaasii* PMS117 colonial forms; (i), PMS117S11241; (ii), PMS117S11242 and (iii), PMS117S11243, (see Fig. 2.4) produced by PMS117S after growth in stationary broth culture.

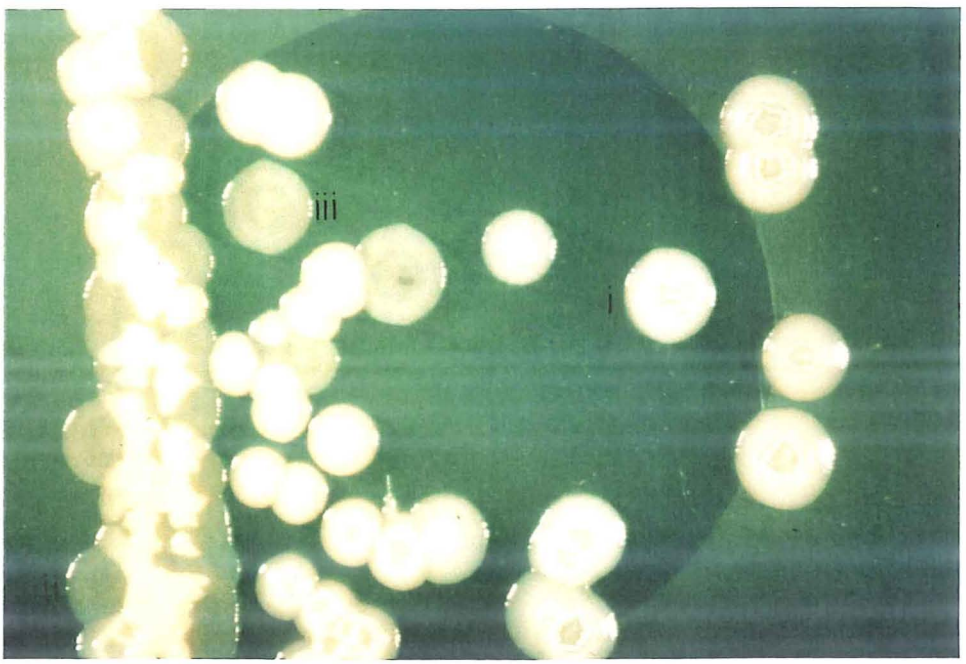


Fig. 2.8. A range of P. tolaasii PMS117 colonial forms; (i), PMS117S1211; (ii), PMS117S1212 and (iii), PMS117S1213, (see Fig. 2.4) produced by PMS117S after growth in stationary broth culture.

findings were reported by Shapiro (1986) who suggested that the maintenance of colony types was indicative of the operation of multicellular controls assuring morphogenetic heredity.

The effect of crowding on colony morphogenesis

Sectoring of smooth colonies on agar media was most prolific in colonies situated near the plate periphery, while colonies toward the centre of the plate remained unsectored (Figs 2.1 and 2.2). This suggested that the degree of crowding may effect the ability of a given colony to undergo morphological differentiation. To test this hypothesis a distinctive colony variant of *P putida* PMS118S, PMS118S13, was inoculated onto a plate of PAF as shown in Fig. 2.9. When the single colony was allowed to grow in relative isolation, it developed the distinctive morphological pattern of its parent, but when surrounded by other colonies, PMS118S13 failed to differentiate and was indistinguishable from the smooth, wild type, PMS118S, colonial form. The inability of colonies growing under crowded conditions to differentiate is possibly related to the availability of non-specific physiological parameters, particularly nutrients, but also pH, oxygen, etc. The presence of a large number of bacterial colonies on an agar plate would rapidly deplete available nutrients and as a result the diameter of centrally located colonies remains small and they do not spread. Colonies near the plate periphery continue to expand in the direction of the unexploited agar and it is during this stage that sectoring and colony differentiation occur.

Active exclusion of one colony by another was sometimes observed. This was particularly apparent in colonies growing near the plate periphery and distinct intercolony boundaries between adjacent colonies were seen. This activity suggests that cells within a colony are able to detect the presence of adjacent colonies.

The ability of *P. putida* PMS118S and *P. tolaasii* PMS117S to produce a range of intricately patterned and regulated colony forms indicates the presence of systems for coordinating the activities of cells within a colony. These systems are not understood, but are thought to enable bacteria to form multicellular aggregates in diverse environments such as soil and water, where the ability to form organized colonies may be essential to the establishment, survival, proliferation and spread of *Pseudomonas* populations in the wild (Shapiro 1986).

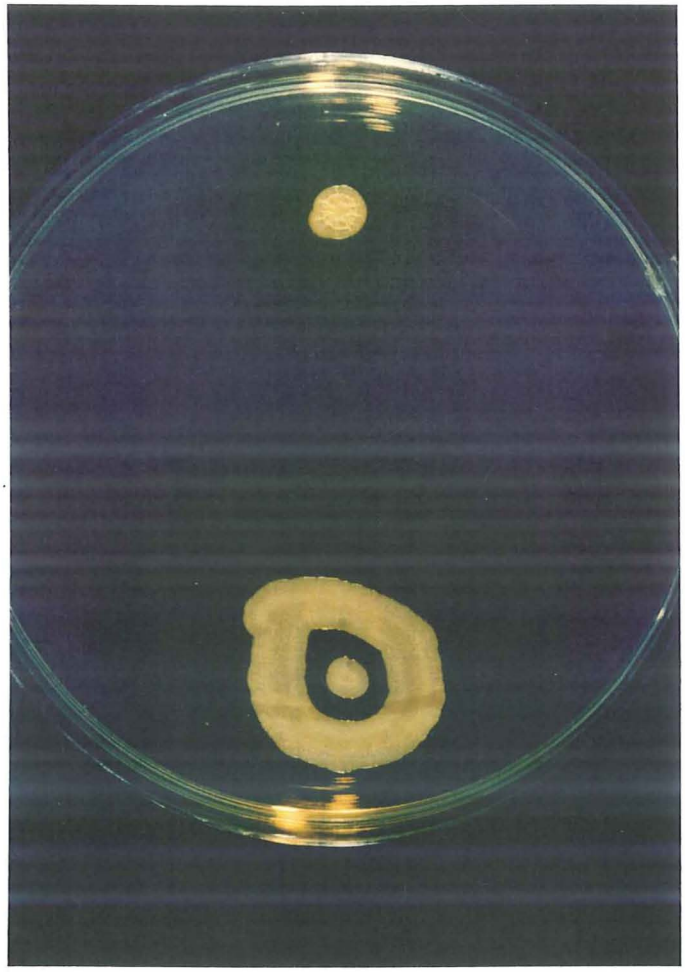


Fig. 2.9. The effect of crowding on colony morphology. Convoluted morphology is not expressed when *P. putida* PMS118S13 is in close association with other colonies.

2.3.3. PRODUCTION OF ROUGH AND SMOOTH COLONIAL FORMS IN PEAT

The production of rough colonial forms of *P. putida* PMS118S and *P. tolaasii* PMS117S was detected in sterile peat. After 3 wk incubation, 50 % of the *P. tolaasii* colonies and 30 % of the *P. putida* colonies were of the rough colonial form. Neither species produced smooth colonies from rough colonies in peat. These results demonstrate that the production of rough forms by *Pseudomonas* isolates is not confined to laboratory media and occurs readily on natural substrates, further highlighting the ecological importance of these colony variants. It would be of value to extend this work and examine the effect of a range of environmental conditions on colony morphogenesis.

2.3.4. THE EFFECT OF STRESS ON SMOOTH AND ROUGH COLONIAL FORMS OF *P. PUTIDA* PMS118 AND *P. TOLAASII* PMS117

Nutrient stress

The growth of smooth and rough colonial forms of *P. putida* PMS118 and *P. tolaasii* PMS117 in KB broth and dilute KB broth is shown in Figs 2.10 and 2.11. There was little difference in the growth of *P. putida* colonial forms in KB, although cells of the 'roughest' form, PMS118Rr, grew to a greater density than PMS118R, which grew to a greater density than the smooth form. After 6 wk, the number of bacteria within each flask had decreased to approximately 2.2×10^7 cfu ml^{-1} and a range of different colonial types were observed after spreading appropriate dilutions on KB. The two *P. tolaasii* colony forms, PMS117S and PMS117R, also grew at similar rates in KB broth, but grew at a slower rate than *P. putida* (Figs 2.12 and 2.13). The number of *P. tolaasii* PMS117 cells of the smooth and rough colonial forms surviving after 6 wk was 5.0×10^6 and 6.2×10^6 cfu ml^{-1}, respectively, and a range of different colonial types were observed after spreading appropriate dilutions from the flask containing the smooth form on KB plates. The rough form remained relatively stable.

In dilute KB broth the growth of *P. putida* PMS118 colonial forms displayed some differences. The number of bacteria of the smooth form and the first derived rough form, PMS118R, declined markedly after inoculation into the dilute broth. The number of bacteria of the smooth form continued to decline during the first 10 h, while the number of PMS118R cells

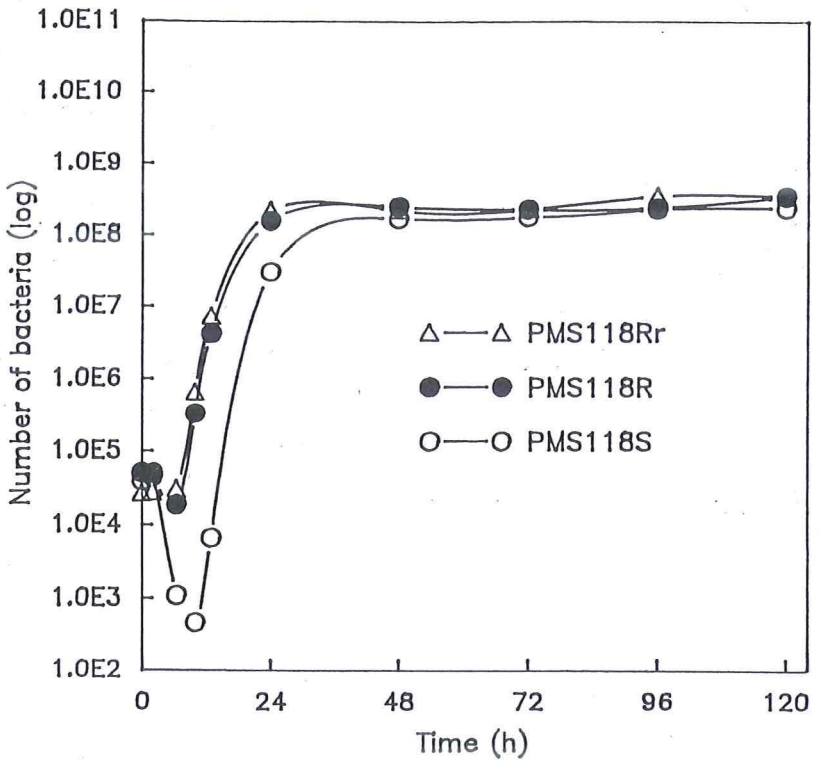


Fig. 2.10. Growth of three *P. putida* PMS118 colonial forms; PMS118S, PMS118R and PMS118Rr, in dilute (1/100 strength) KB broth.

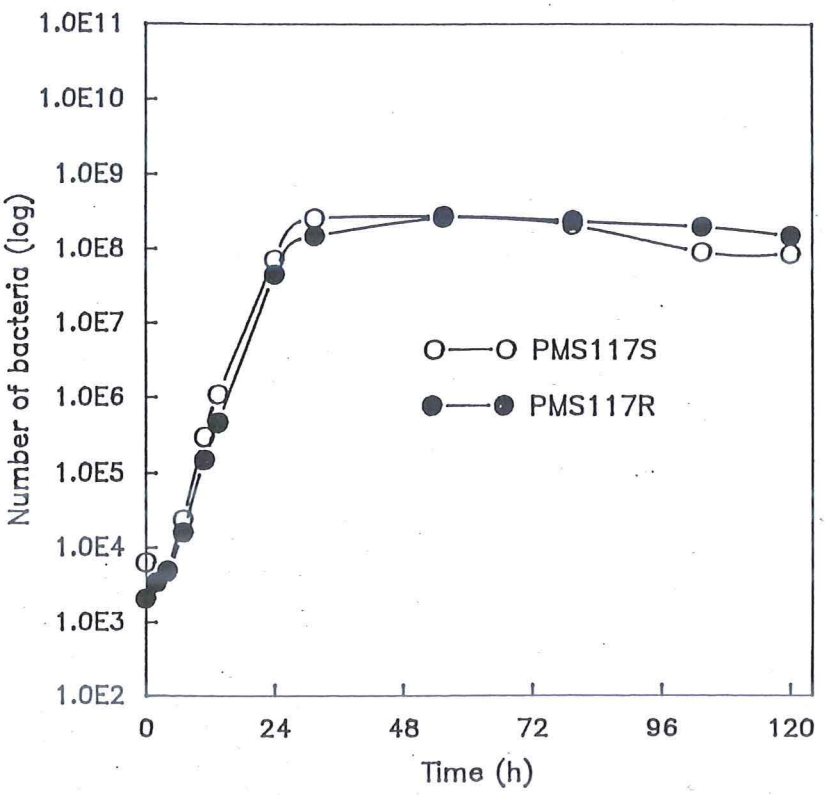


Fig. 2.11. Growth of two *P. tolaasii* PMS117 colonial forms; PMS117S and PMS117R, in dilute (1/100 strength) KB broth.

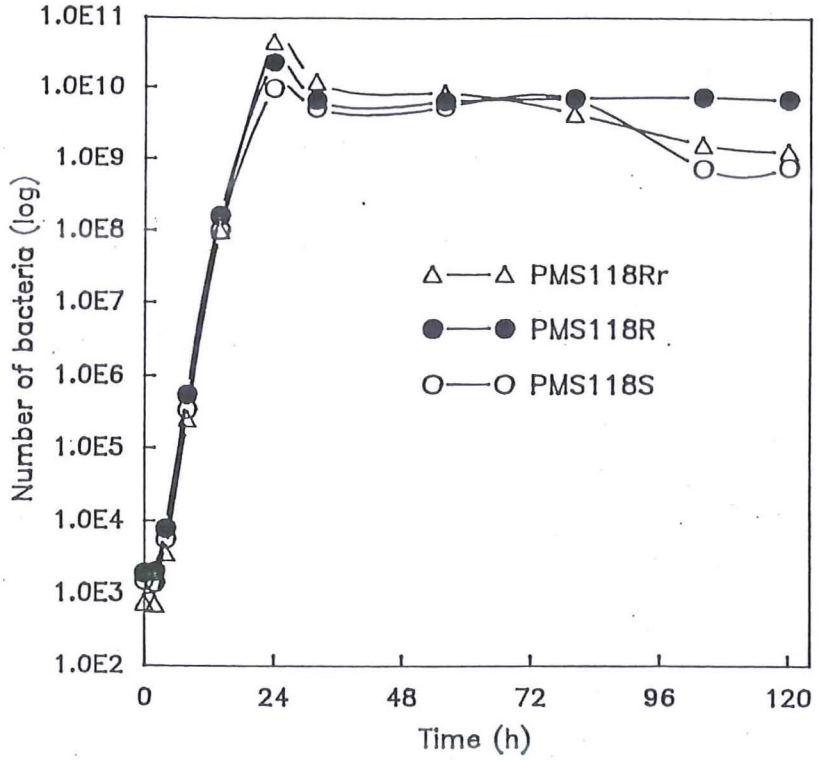


Fig. 2.12. Growth of three *P. putida* PMS118 colonial forms; PMS118S, PMS118R and PMS118Rr, in full strength KB broth.

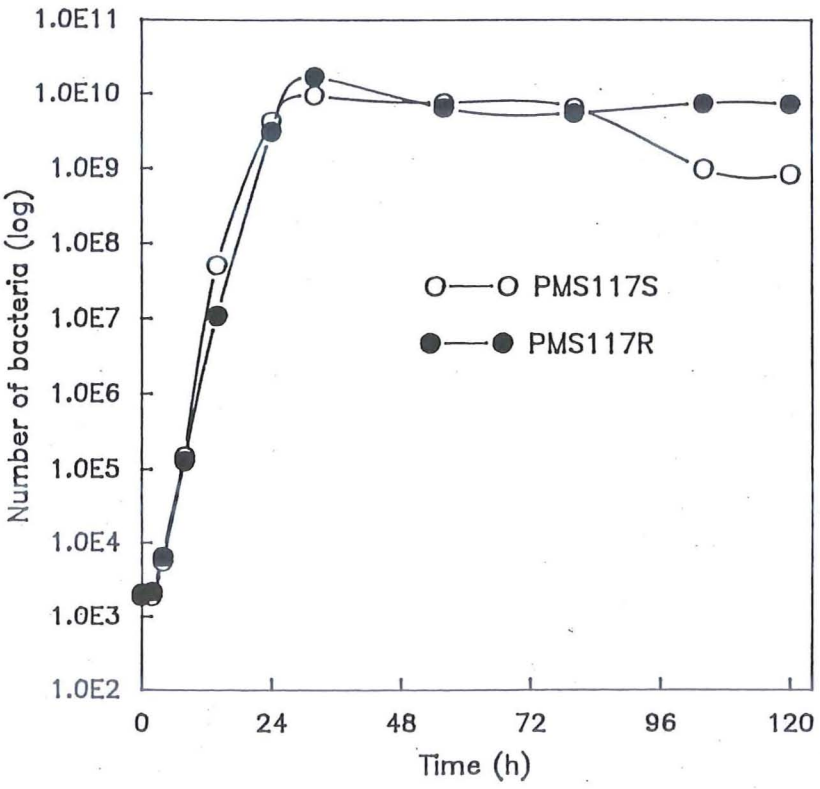


Fig. 2.13. Growth of two *P. tolaasii* PMS117 colonial forms; PMS117S and PMS117R, in full strength KB broth.

increased after an initial 6 h period of decline. PMS118Rr cells, while showing a 6 h lag phase, did not decline in numbers once inoculated into the dilute media. The growth rate of each colony form during the exponential phase was very similar and was almost as rapid as the growth of the cells in full strength KB. All colonial forms reached a density of approximately 4.0×10^8 cfu ml^{-1} within 48 h and after 8 wk in dilute broth the number of bacteria within each flask had not declined and the colony morphology of the different forms remained true to the original.

Cells of the smooth form of *P. tolaasii* PMS117 also displayed a decline in numbers after inoculation into dilute KB broth and this was not detected in the rough form. Growth of *P. tolaasii* (irrespective of colonial form) during the exponential phase, in dilute broth, was slower than in full strength KB and was also slower than *P. putida* in either dilute or full strength broth. The number of bacteria surviving after 8 wk was the same as after 48 h and both forms were stably maintained.

These results show that all isolates were able to grow well and multiply rapidly under nutrient limited conditions, but the rough forms were better able to withstand the shock which resulted from the transfer of cells from a nutrient rich to a nutrient poor environment. This ability may be due to the altered lipopolysaccharide layer of the rough colonial forms which may provide increased resistance to osmotic shock which would have been a major stress factor in the transfer of cells to the dilute broth. The more rapid response of the rough forms to the nutrient poor environment suggests that these forms may possess a more efficient metabolic system, and/or may be able to more readily scavenge limiting nutrients.

In dilute broth cultures, nutrients would be rapidly exhausted and therefore multiplication of cells would not occur (Postgate 1976). Some bacteria are able, under conditions of low exogenous nutrients, to differentiate, forming structures, such as spores and cysts, which ensure long term survival. Pseudomonads are unable to form resting structures (Palleroni 1984), nevertheless, they are able, as this work demonstrates, to remain viable under conditions of starvation for long periods of time. Many non-spore forming bacteria possess this ability (Gentry *et al.* 1971, Dawes 1976) and is usually achieved by lowering the rate of metabolic activity and

relying on endogenous metabolism of cellular constituents and storage polymers, and efficiently scavenging exogenous substrate molecules (Kjelleberg *et al.* 1987).

The reason for the stability of isolates under conditions of nutrient limitation is not known, but may result from the scarcity of certain factors necessary for differentiation of colonial variants. Advantage was taken of the stability and viability of cells in dilute broth culture and dilute KB broth was used as a storage medium for *Pseudomonas* isolates. The following method was used:- An inoculating loopful of cells was removed from a plate of KB after overnight culture and suspended in 1 ml of 1/50 strength KB contained within a 1.5 ml Eppendorf tube, the tube was labelled and stored at room temperature. Isolates stored in this manner have remained highly viable and stable, for more than 20 mth.

Resistance to UV light

The different colonial forms of each species showed markedly different tolerances to UV light (Figs 2.14 and 2.15). In *P. putida* PMS118, the 'rougher' the colonial form, the greater its resistance to the mutagen. This trend was reversed in *P. tolaasii* PMS117 and both colony forms displayed a sensitivity to UV light which was much greater than that shown by the smooth form of *P. putida* PMS118, the most sensitive of the *P. putida* PMS118 colonial forms.

The increased resistance of the rough colonial forms of *P. putida* PMS118 to UV light most likely reflects the altered cell wall material which may reflect a greater amount of UV light. However, it is also possible that the rough colonial forms of *P. putida* PMS118 may possess more effective DNA repair systems. These systems are able to reverse damage caused to DNA by mutagens such as UV light (see Walker 1984). The reason for the greater sensitivity to UV light of the rough colonial form of *P. tolaasii* PMS117 is not known, but, may once again be related to the lipopolysaccharide wall layer.

The marked sensitivity of *P. tolaasii* PMS117 to UV light may partly explain why it is not found in soils, or associated with plants. It would be interesting to examine the survival of *P. tolaasii* in sunlight and to determine the effect of the toxin on plants.

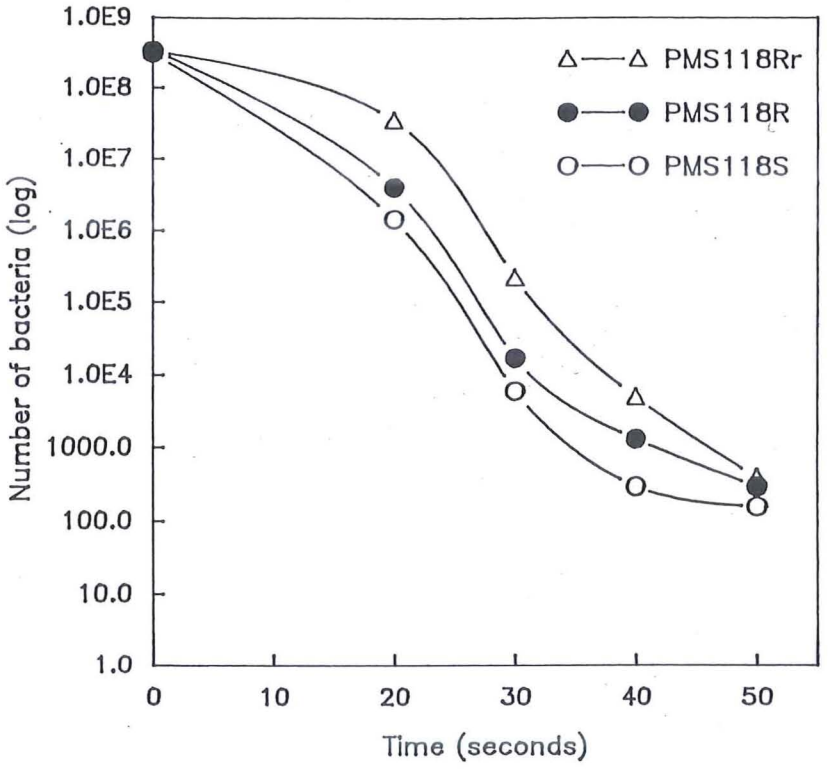


Fig. 2.14. The effect of UV light on the survival of three *P. putida* PMS118 colonial forms; PMS118S, PMS118R and PMS118Rr.

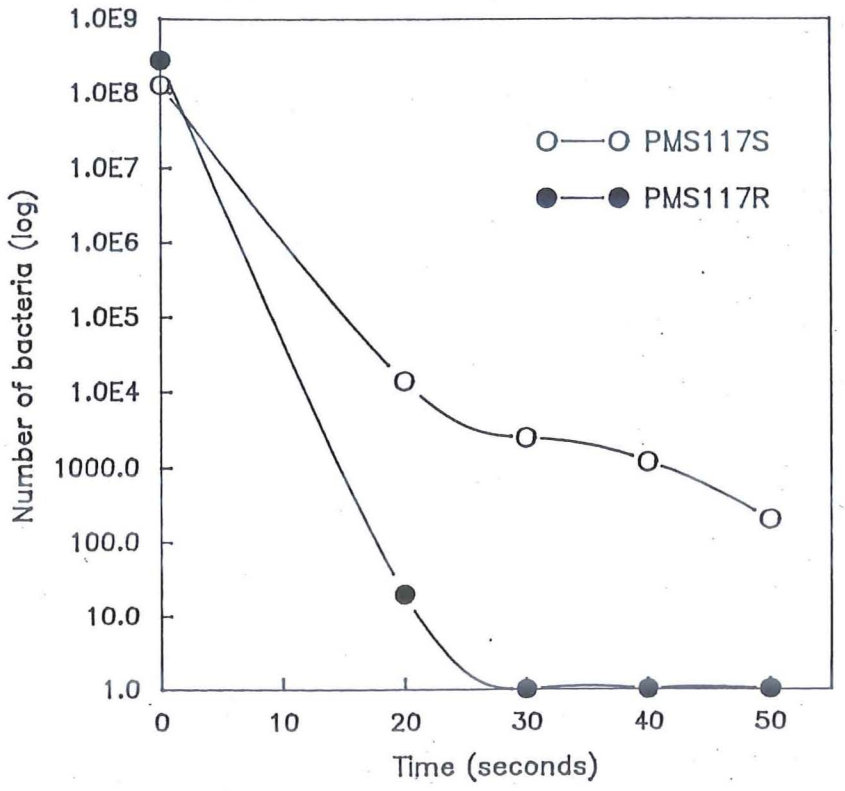


Fig. 2.15. The effect of UV light on the survival of two *P. tolaasii* PMS117 colonial forms; PMS117S and PMS117R.

Siderophore production

The absorption spectra of the pigment-containing supernatant from *P. putida* PMS118S and *P. tolaasii* PMS117S cultures in succinate broth (pH 7.0) are shown in Figs 2.16 and 2.17. The spectra each exhibit two main peaks, at 230 and 400 nm. The absorption spectra of the supernatant from the two species containing the Fe^{3+}-pigment complex also shows two maxima at 230 and 400 nm (Figs 2.16. and 2.17.). These results demonstrate that the pigment maybe a spectral siderophore which has essentially the same properties as the purified siderophore pyoverdine (Meyer & Abdallah 1978), pseudobactin (Teintze *et al.* 1981), and pseudobactin 358 (Marugg *et al.* 1985).

The rough colonial forms of both *P. putida* PMS118 and *P. tolaasii* PMS117 produced greater amounts of siderophore than the smooth forms (Figs 2.18 and 2.19). The number of cells within each flask at the end of the 48 h incubation period was similar between colony forms of each species; 6.0×10^9, 5.5×10^9 and 5.8×10^9 c.f.u. ml^{-1} for PMS118S, PMS118R and PMS118Rr respectively and 9.5×10^8 and 8.5×10^8 c.f.u. ml^{-1} for PMS117S and PMS117R, respectively. This indicates that siderophore production by cells of the rough colonial forms was greater than that of the smooth colonial forms after 24 h and did not occur as a result of the presence of a greater number of cells.

These results show that in environments where iron is limiting, cells of the rough colonial forms are able to produce greater amounts of iron binding compounds than cells of the smooth colonial form. This would most likely provide cells of the rough colonial forms with an ecological advantage.

Respiration

The evolution of carbon dioxide by smooth and rough colonial forms of *P. putida* PMS118 and *P. tolaasii* PMS117 is shown in Figs 2.20 and 2.21. Under iron limiting conditions, cells of the rough colonial forms respired at a greater rate than cells of the smooth forms (Fig. 2.20). In succinate broth supplemented with $FeCl_3$ there was little difference in the rate of respiration of the *P. putida* PMS118 colonial forms (Fig. 2.21), however, differences were still apparent between the

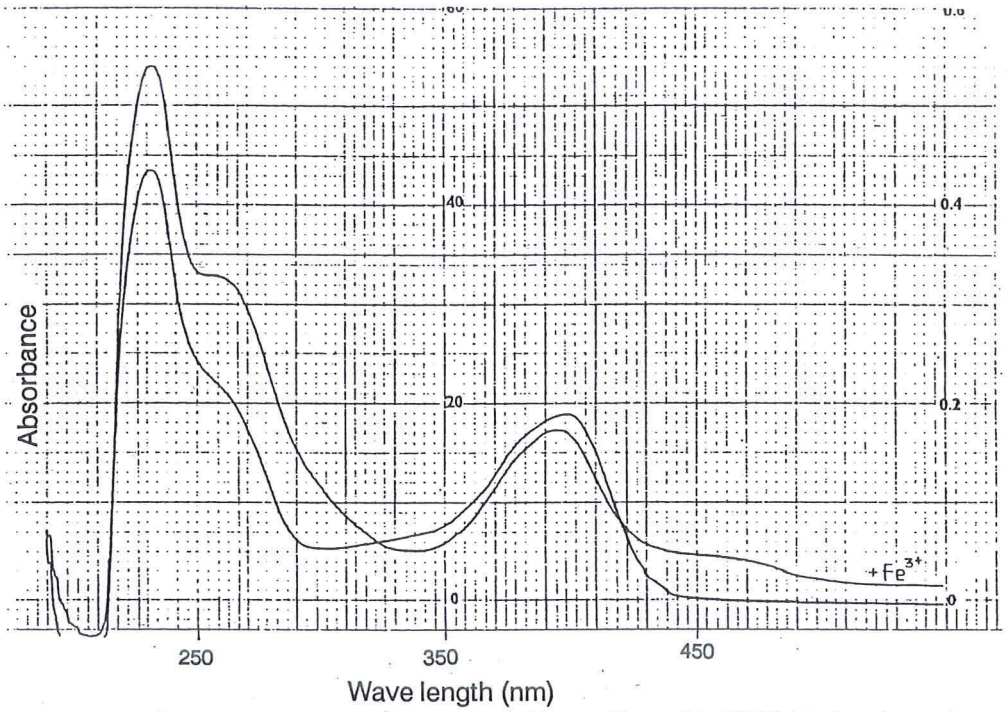


Fig. 2.16. Absorption spectra of the supernatant from a *P. putida* PMS118S culture (grown in succinate salts) containing the free siderophore and the ferric-siderophore complex (after addition of 500 μM $FeCl_3$ to the supernatant).

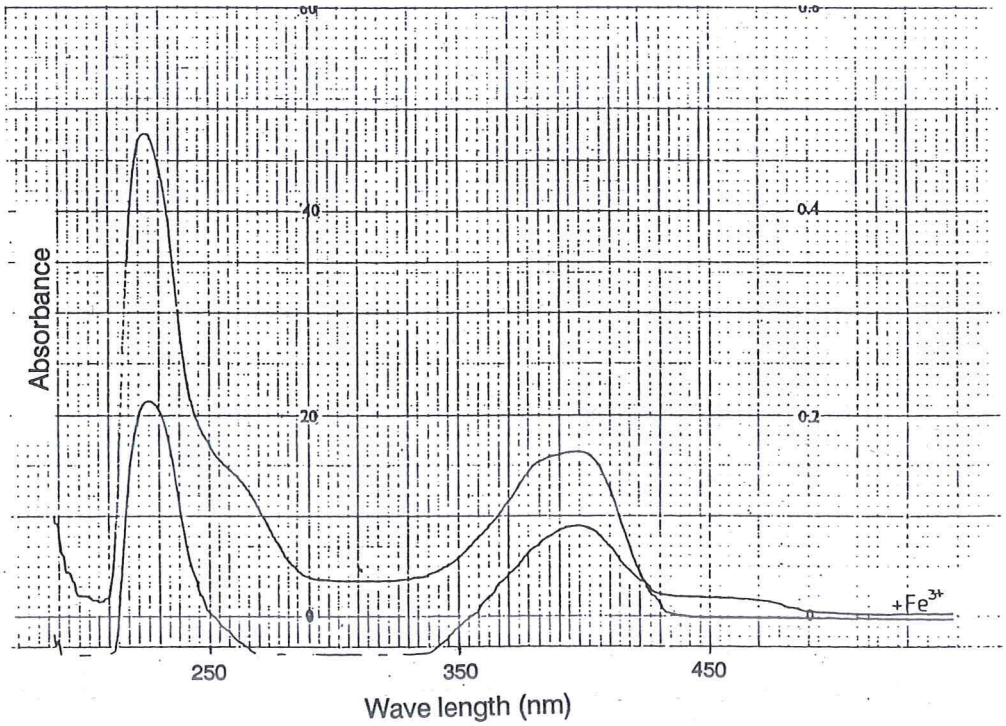


Fig. 2.17. Absorption spectra of the supernatant from a *P. tolaasii* PMS117S culture (grown in succinate salts broth) containing the free siderophore and the ferric-siderophore complex (after addition of 500 μM $FeCl_3$ to the supernatant).

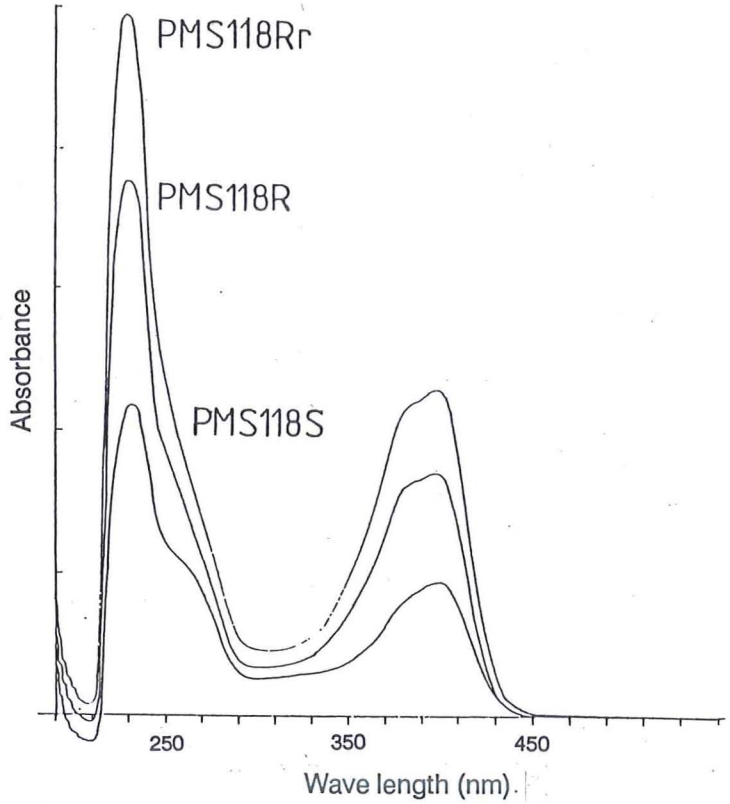


Fig. 2.18. Siderophore production by three *P. putida* PMS118 colonial forms; PMS118S, PMS118R and PMS118Rr, after 48 h growth in succinate salts broth.

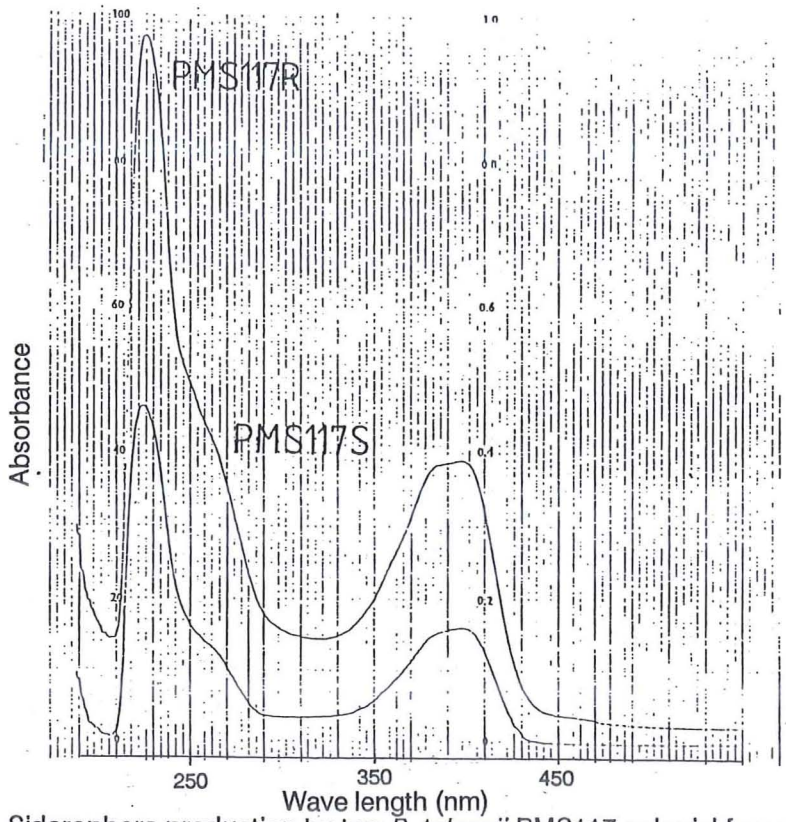


Fig. 2.19. Siderophore production by two *P. tolaasii* PMS117 colonial forms; PMS117S and PMS117R, after 48 h growth in succinate salts broth.

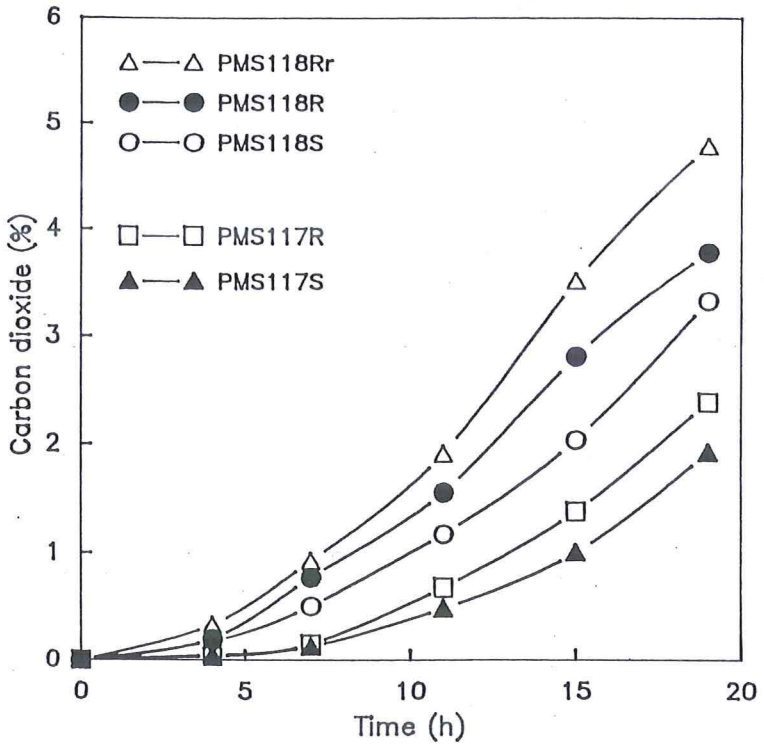


Fig. 2.20. Evolution of carbon dioxide by three colonial forms of *P. putida* PMS118; PMS118S, PMS118R and PMS118Rr, and two colonial forms of *P. tolaasii* PMS117; PMS117S and PMS117R, following growth in iron deplete succinate salts broth.

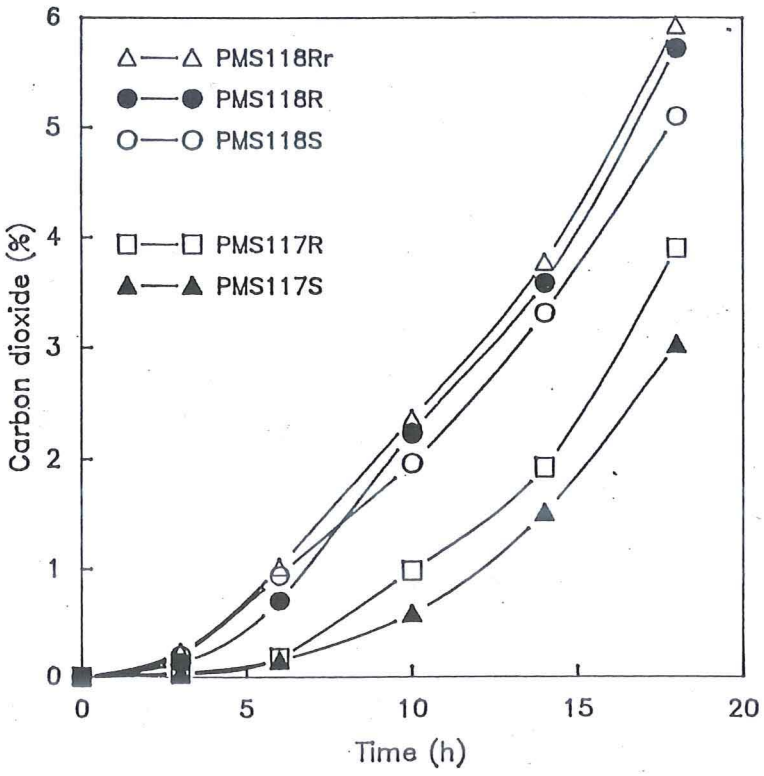


Fig. 2.21. Evolution of carbon dioxide by three colonial forms of *P. putida* PMS118; PMS118S, PMS118R and PMS118Rr, and two colonial forms of *P. tolaasii* PMS117; PMS117S and PMS117R, following growth in iron replete succinate salts broth.

smooth and rough forms of *P. tolaasii* PMS117. The rate of respiration was consistently greater in *P. putida* PMS118 (irrespective of the colonial form), than in *P. tolaasii* PMS117.

These results are interesting, but interpretation without further work requires caution, nevertheless, they do indicate that cells of the rough colonial forms are better adapted to survive under nutrient stressed conditions. In unamended succinate salts broth, growth is limited by the scarcity of iron. In order to acquire this essential element the isolates must synthesize and export a siderophore, import the ferric-siderophore complex and remove ferric iron from the chelating agent. This process requires energy (Neilands 1982) which is provided by metabolism of nutrients within the broth. The greater rate of respiration by cells of the rough colonial forms indicates that, unless respiration is uncoupled, these cells have more energy to utilize. Some of this energy appears to be directed toward the production of the siderophore (see above) and cells which can produce large amounts of this compound are likely to possess a survival advantage in the natural environment. This result raises many questions concerning the control of metabolism within the different colonial forms and a closer examination of the respiration and energy budgets of these colonial forms would be interesting.

Motility

Cells of the rough colonial forms of both *P. putida* PMS118 and *P. tolaasii* PMS117 swarmed further on semi-solid agar than cells of the smooth colonial forms. After 24 h the colony diameter of smooth *P. putida* was 4 mm, while that of PMS118R was 16 mm. Cells of the smooth colonial form of *P. tolaasii* had formed a colony 9 mm diam and the diameter of the rough colony was 12 mm.

This result may be attributable to differences in the lipopolysaccharide layer of the rough forms which may enable more rapid passage through moist environments, but may also reflect the greater rate of respiration of the rough forms (see above). The more rapid utilization of nutrients by the rapidly respiring rough cells would lead to the development of a steeper nutritional gradient within the agar medium, than that created by cells of the smooth forms, and the response of the rough forms to this self created gradient could explain the observed result. Irrespective of the reason, the ability to rapidly utilize nutrients and then move quickly in the

direction of unexploited regions, would undoubtedly provide cells of the rough colonial forms with a distinct ecological advantage (see Chapter 3.0.).

This set of experiments provides evidence which indicates that cells of the rough colonial forms are better adapted to withstand stress. The ability to produce stress-tolerant forms may be an important factor governing the survival and proliferation of bacteria within the competitive, nutrient-limited, mushroom casing layer.

Further investigation of rough colonial forms may enable selection of 'super' stress resistant cells. Such bacteria would be useful vehicles for transport of engineered genes into the natural environment, where survival is of prime importance.

CHAPTER THREE

ECOLOGICAL ASPECTS OF THE INTERACTION BETWEEN *AGARICUS BISPORUS* AND *PSEUDOMONAS PUTIDA*

3.1. INTRODUCTION

3.1.1 BACTERIAL ECOLOGY OF THE CASING LAYER

A. bisporus mycelium grows in close association with other micro-organisms, both within the compost (Stanek 1972, 1976) and within the casing layer (Hayes *et al.* 1969). Several studies have examined the bacterial ecology of this environment and have demonstrated a significant link between volatile metabolites produced by mycelium growing on compost and the occurrence of pseudomonads in the casing layer which are associated with fruit body initiation (Hayes *et al.* 1969, Hayes 1972, Hayes & Nair 1976).

Cresswell & Hayes (1979) examined the initial stages of colonization of peat casing soils with aerobic bacteria and reported that both the number of bacteria and composition of the population fluctuated during a cropping cycle. They reported that pseudomonads were the dominant group of bacteria within this environment (see Chapter 2.0.) and found that the total number of bacteria within the casing layer increased one thousand fold after application of the casing soil to the colonized compost. They also found that the number of bacteria peaked at the time of the first flush at between 10^8 and 10^9 cfu per fresh weight gram of casing soil and then declined until the third flush, where a second peak was observed. Doores *et al* (1987) and Samson *et al.* (1987) reported similar trends, but found that the decline in the number of bacteria after the first flush continued throughout the remainder of the cropping cycle.

These studies suggest a close relationship between the fluorescent pseudomonads and *A. bisporus*:- The fungus appears to influence the bacterial flora of the casing layer causing members of the genus *Pseudomonas,* particularly those of the *P. putida*, *P. fluorescens* group, to dominate (Chapter 2.0.). In turn, these pseudomonads affect the growth and development of the mushroom; some promoting fruit body initiation and others causing disease (Chapter 4.0.).

The effect that these pseudomonads have on the growth of *A. bisporus* may take place at a distance, as is found with *Bacillus subtilis* when it effects sclerotium formation of *Rhizoctonia solani* (Henis and Inbar 1968), or may require direct contact as does *Trichoderma harzianum* with *Sclerotium rolfsii* in order to induce strand formation (Hadar *et al.* 1981). The studies of

Preece and Wong (1982) revealed that *P. tolaasii*, isolated from the casing layer, was firmly attached to *A. bisporus* hyphae and a scanning electron microscope study of mycelium in the casing layer, conducted by Masaphy *et al.* (1987), found many bacteria attached to the hyphal surfaces. Lockwood (1968) also reported adhesion of bacteria to hyphal surfaces and Fradkin & Patrick (1985) observed bacteria attached to the surfaces of fungal propagules.

Hume and Hayes (1972) reported that fruit body initiation of *A. bisporus* did not occur until the mycelium had made contact with the bacterial colony. Similar results are presented in Chapter 4.0. and show that basidiome initiation is confined to the area immediately above the bacterial colony. These results indicate that contact between *P. putida* and *A. bisporus* mycelium is essential for fruit body initiation to occur and suggests that adhesion of *P. putida* to the mycelium may be an important part of the fruit body initiation process. It is conceivable that *A. bisporus* actively encourages *P. putida* to associate with its hyphal surfaces through the production of compounds which attract the bacterium.

3.1.2. CHEMOTAXIS

Chemotaxis, the ability of a motile bacterium to respond to changes in its chemical environment by altering its pattern of motility, is a common attribute of many bacteria. This behavioural response provides bacteria with a means of locating nutrients and avoiding harmful environments (Chet & Mitchell 1976). Chemotaxis plays an important role in microbial interactions and in the colonization of the surfaces of living organelles, such as plant roots, (Currier & Strobel 1977, Gitte *et al.* 1978, Heinrich & Hess 1985, Bashan 1986, Gafny *et al.* 1986, De Weger *et al.* 1987), seeds (Scher *et al.* 1985) and fungal propagules (Arora *et al.* 1983, Lim & Lockwood 1988). Chemotaxis increases the chances of a bacterium contacting a nutritionally favourable surface and chemotaxis alone is thought to be sufficient to hold a bacterium at a mucilaginous surface (Freter *et al.* 1981).

Porous, water saturated, nutrient deplete environments, such as the mushroom casing layer, provide ideal conditions for bacterial migration (Griffin & Quail 1968, Madsen & Alexander 1982, McCoy & Hagedorn 1979). Chemotactic bacteria which exist in such habitats have been shown to possess a survival advantage over non-chemotactic organisms (Ames & Bergman

1981, Lauffenburger *et al.* 1981), because of their ability to locate nutritionally favoured surfaces on which to attach.

Soil inhabiting, fluorescent pseudomonads, are known to exhibit chemotaxis toward a range of chemical stimuli including sugars (Lynch 1980), amino acids (Scher *et al.* 1985), organic acids, (Cuppels 1988) and aromatic acids (Harwood *et al.* 1984). Hyphal and spore exudates have been shown to provide substrates for bacteria (Lockwood 1968, Siala & Gray 1974) and *A. bisporus* mycelium is known to produce compounds which can be used by pseudomonads for growth (Hayes *et al.* 1969, Hayes & Nair 1976). Exudates of *Pythium debaryanum* mycelium strongly attracted a predatory fluorescent pseudomonad (Chet *et al.* 1971) and exudates of fungal propagules served as attractants for *P. putida* and *P. fluorescens* (Arora *et al.* 1983, Lim & Lockwood 1988). It is likely that *A. bisporus* mycelium may also exude compounds which serve as attractants for bacteria.

3.1.3. ADHESION OF BACTERIA TO LIVING SURFACES

The ability of bacteria to adhere to surfaces, both animate and inanimate, has been known for many years, but it is only in recent times that the importance of bacterial adhesion has been appreciated (Jones 1977).

Some bacteria possess the ability to influence the growth and development of other organisms, but this ability is frequently dependent upon effective adherence to surfaces of the 'host' organism. Colonization of legume roots by *Rhizobium* sp. is necessary before the bacterium can infect the host cells, enter the root cortex and incite the formation of nitrogen fixing root nodules (Dazzo 1984). Similarly, colonization of dicot root surfaces is necessary before *Agrobacterium tumefaciens* can infect its host (Lippincott & Lippincott 1969, Matthysse *et al.* 1978) and colonization of plant root surfaces by rhizobacteria, such as *P. putida* and *P. fluorescens*, must occur before subsequent effects of the bacteria on promotion, or limitation of plant growth can take place (Schroth & Hancock 1982, de Weger *et al.* 1987). In addition, adhesion and colonization of animal tissues by bacterial pathogens has been shown to be an important part of the infection process (Walker & Nagy 1980) and adherence of oral bacteria to teeth is necessary for the development of a range of dental diseases (Orstavik 1980).

Mechanisms of adhesion

Adhesion can be either specific, requiring some form of stereochemical constraint, or non-specific. Non-specific adhesion is less readily definable, but is equally important in adhesion of micro-organisms to living surfaces (Rutter 1984). Where non-specific adhesion occurs, a range of different interactions may be involved, including electrostatic and hydrophobic interactions, hydrogen bonds and London-van der Waals interactions. Specific adhesion involves contact between neighbouring interacting groups on the micro-organism and substratum and involves a lock-and-key mechanism (see Rutter 1984).

Interactions involved in adhesion

Bacteria, eukaryotic cells and other surfaces to which bacteria adhere possess an overall negative charge (Jones 1977). The DLVO theory (Rutter & Vincent 1980) is most frequently used to described adhesion between two negatively charged surfaces and predicts that attachment is a two phase, time dependent phenomenon. The initial phase is an instantaneous, reversible adsorption stage where bacteria are held at a finite distance from the substratum by a balancing of London-van der Waals attraction forces and electrostatic repulsion forces. The second phase which leads to firm attachment is a time dependent, irreversible adherence phase. During this stage bacteria are thought to overcome the short range repulsive forces by synthesis of adhesive, extracellular polymers which enable the bacteria to come within 1 nm of the surface. At this distance attractive forces are greater than repulsive forces, leading to firm, irreversible attachment.

Support for this theory was provided by Marshall *et al.* (1971) who showed that a marine bacterium could be easily washed off a substratum during the first phase of attachment, but after the build-up of adhesive polymers, became firmly attached. Matthysse (1986) detailed a two phase process in the attachment of *A. tumefaciens* to carrot cells, the second stage of which involved the production of cellulose fibrils which anchored the bacterium tightly to the cell surface. A two phase process has also been described in the attachment of *Rhizobium* to legume root hair surfaces (Dazzo & Truchet 1983, Dazzo 1984).

In contrast to a two phase adhesion process, James *et al.* (1985) reported rapid firm adhesion of bacteria to raddish root surfaces and a similar single phase adsorption process has been reported by other authors (see Fletcher 1980). Fletcher (1980) argued that instantaneous adhesion can still be explained by the DLVO theory; some bacterial surfaces are complex and comprise several layers of preformed extracellular polymers (Sutherland 1980). Rough, uneven surfaces of this type cause the repulsive forces between the bacterium and substratum to be significantly reduced, enabling firm adsorption to occur rapidly.

Divalent cations, including calcium and magnesium, have been shown to promote the binding of *P. tolaasii* to barley roots (Nissen 1973) and of *P. fluorescens* to radish roots (James *et al.* 1985). It is thought that the divalent ions mediate adsorption by bridging negatively charged groups on the bacterial surface, with negatively charged groups on the surface of the plant tissue (Nissen 1973).

Hydrophobic interactions are also involved in the adhesion of bacteria to surfaces (Rosenberg & Kjelleberg 1986) and several studies have shown a correlation between the hydrophobicity of cell surfaces and the ability of bacteria to colonize negatively charged surfaces (Fletcher & Loeb 1979, Lindahl *et al.* 1981, Loosdrecht *et al* 1987). Hydrophobic interactions allow bacteria to approach negatively charged surfaces close enough to enable interaction of more specific binding molecules (Ofek & Beachy 1980).

In contrast to non-specific adhesion, the association between *Rhizobium* spp. and its legume host involves specific carbohydrate receptors on the bacterial surface which interact with a specific multivalent lectin (trifoliin A) associated with the root hair surface (Dazzo & Brill 1979). *P. putida* has also been shown to interact in a specific manner with a glycoprotein isolated from root surfaces of *Phaseolus vulgaris* which causes agglutination of the bacterium (Anderson 1983, Tari & Anderson 1988). The adherence of *P. putida* to the agglutinin is thought to represent a primary recognition event which Anderson *et al.* (1988) suggested may be important in securing the initial niche.

Structures aiding adhesion

Some bacteria possess specialized structures on their surfaces, such as fimbriae (pili) (Jones & Isaacson 1983) and ploymeric accumulations known as fibrillae, but which are frequently referred to as 'blebs', 'droplets', or fibrils (Corpe *et al.* 1976). These structures enable bacteria to make contact with cell surfaces, facilitating the onset of phase II of the attachment process by bridging the space between the adsorbed microbe and the surface (Jones & Isaacson 1983). Flagella are also thought to play a role in adhesion by providing forward motion which under some conditions may be sufficient for the bacterium to overcome the short range repulsive forces and adhere to the surface of a given substratum (Uhlman & Jones 1982). Flagella have also been reported to adhere directly to surfaces, performing a function similar to fimbriae (Meadows 1971).

Bacteria, particularly those in aquatic environments, such as the aqueous phase of soil, usually do not possess specialized structures for attachment and most are thought to adhere by non-specific means, facilitated by the production of extracellular polymeric adhesives (Fletcher 1980). These polymers are usually synthesized after attachment and have been shown to be predominantly polysaccharide (Corpe *et al.* 1976, Read & Costerton 1987). Some evidence exists which shows that synthesis of capsular polysaccharides by attached bacteria can actually lead to detachment (Rosenberg *et al.* 1983). Capsulated bacteria have also been shown to be less hydrophobic than enzymatically decapsulated bacteria and to adhere poorly to hydrocarbon (Rosenberg *et al.* 1983).

The effect of environmental conditions on attachment properties.

The adhesive properties of a bacteria are affected by a range of physiological and environmental conditions (Fletcher 1980). Small starved bacteria, which are frequently found in aquatic and soil environments, have an increased tendency for firm adhesion (Marshall *et al.* 1971, Kjelleberg *et al.* 1983) and bacteria grown under carbon or nitrogen limitation have significantly altered adhesion patterns (Brown *et al.* 1977). The physico-chemical properties of the potential attachment surface have also been shown to affect adhesion (Fletcher & Loeb 1979).

The advantages of adhesion

Bacteria attached to surfaces benefit from the association in many ways. In aqueous environments of low nutrient availability, such as some soils, a variety of low molecular weight species including sugars, amino acids, biogenic salts and inorganic ions, are attracted to the negatively charged surfaces. Bacteria which attach to these surfaces are able to take advantage of the nutrient enriched state of the solid-water interface (Fletcher 1984). Bacteria attached to living surfaces benefit in additional ways from the secretions of the host cells and utilization of the products of extracellular activity of the host cell enzymes (Jones 1977). Furthermore, bacteria attached to surfaces are less likely to be removed from their particular environment

3.1.4 STUDY AIMS

Two previously unexplored aspects of the ecology of the interaction between *P. putida* and *A. bisporus* mycelium were examined. The response of the bacterium to mycelial exudates was investigated and an examination of attachment of *P. putida* to hyphal surfaces was made. These studies were conducted in order to gain evidence from an alternative perspective for the involvement of *P. putida* in fruiting of *A. bisporus*. Were *P. putida* repelled by mycelial exudates, then it would be difficult to see how the bacterium could be involved in the fruiting process. Similarly, if the bacterium did not colonize the hyphal surfaces then it would be difficult to explain how, in a water saturated environment, the bacterium could provide the fungus with the reproductive stimulus.

P. tolaasii, the causal organism of brown blotch disease (Chapter 2.0.) was included in the study as a 'control' organism and its adhesive and chemotactic properties were examined alongside those of *P. putida.* Also included in the studies were the commonly produced rough colonial forms of both species, as the differences in their lipopolysaccharide layer (see Chapter 2.0.) may have enabled useful comparisons to be made.

3.2. MATERIALS AND METHODS

3.2.1. STRAINS

A commercial *A. bisporus* hybrid strain, 'Horst U3' (Somycel) was used throughout. Bacterial strains are listed in Table 3.1. Bacteria were grown at 28 °C and *A. bisporus* U3 was grown at 25 °C, unless otherwise stated.

Table 3.1. Bacterial strains.

Strain	Characteristics	Reference/source
P. putida		
PMS118S	Smooth colonial form, Bas^{+a}	This study (Chapter 2.0.)
PMS118R	Rough colonial form, Bas^{+}	This study (Chapter 2.0.)
P. tolaasii		
PMS117S	Smooth colonial form Bas^{-}	This study (Chapter 2.0.)
PMS117R	Rough colonial form, $Bas^{-/+}$	This study (Chapter 2.0.)
P. aeruginosa		
OT15		Department of Microbiology, Otago University, N.Z.
P. syringae pv *syringae*		
PDDCC7607	Ice nucleating strain	Plant Disease Division Culture Collection, D.S.I.R., Auckland, N.Z.

[a] Bas^{+}, promotes basidiome initiation of *A. bisporus*; $Bas^{-/+}$, variable basidiome promoting ability; Bas^{-}, inhibits basidiome initiation

3.2.2. CHEMOTAXIS

In vitro chemotaxis assay

Media, culture conditions and preparation of bacteria for in vitro *chemotaxis assay*

Pseudomonas cultures were enriched for actively motile cells before conducting chemotaxis assays (Adler 1973) by allowing cells to swarm on 1/10 strength KB, solidified with 0.3 % agar.

Bacterial isolates were grown overnight in nutrient broth under vigorous aeration. Fresh medium (10 ml) was inoculated with this culture to an initial A_{550} of 0.05 and then re-incubated

with vigorous shaking until the A_{550} reached 0.28. At this density the bacterial population was growing exponentially. The cells were harvested by centrifuging for 5 min at room temperature (4000 x *g*, 4 °C) and washed twice with cold (4 °C) 10 mM phosphate buffer (pH 7.0) and resuspended in 10 ml of chemotaxis buffer (10 mM phosphate buffer (pH 7.0) containing 10^{-4} M EDTA) (Adler 1973) to a density of 5×10^7 cells ml^{-1}. Bacteria were checked microscopically for motility before performing an experiment.

Media, culture conditions and preparation of fungal exudates for in vitro *chemotaxis assay*

Growth of *A. bisporus* in preparation for exudate collection was in 250 ml flasks containing 20 ml of CMM broth. The flasks were incubated without shaking for 14 days, by which time the fungal mat had colonized the surface of the medium.

Exudates were prepared using a method similar to that described by Chet *et al.* (1971). The fungal mat was washed carefully, five times, under sterile conditions, in 10 mM phosphate buffer (pH 7.0) by pouring 100 ml of buffer into the flask and gently agitating for 10 min. After the final wash, the mycelial mat was incubated for 8 h without shaking, at 25 °C, in 50 ml of 10 mM phosphate buffer (pH 7.0). The buffer containing exudates of *A. bisporus* mycelium was then passed through an 0.22 μm Millipore filter and stored in the dark at 4 °C and used as a source of chemotactic substances. For diagnostic purposes, the exudate was autoclaved (15 min, 120 °C, 1.2 atm) or dialyzed (10,000 MW exclusion tubing) against distilled water for 24 h at room temperature.

In vitro *chemotaxis assay*

The assay system used to quantify the chemotactic response was essentially that described by Adler (1973). A U-shaped glass tube (3 mm diam) was placed between a clean sterile glass slide and a cover slip, forming a small chamber into which a suspension of bacteria in chemotaxis buffer was introduced. The attractant was drawn into a sterile 1 μl glass capillary tube which had been sealed at one end by heating and the capillary tube plus attractant were introduced into the bacterial suspension within the chamber. Bacteria were exposed to the attractant for 30 min, after which time the capillary tube was removed from the chamber, rinsed in 70 % ethanol, then in

water and broken at the sealed ends. The contents were ejected, using a modified Pasteur pipette bulb, into 1 ml of 10 mM phosphate buffer pH (7.0) and appropriate dilutions made. The number of bacteria contained within the capillary tube was determined using the counting method of Miles and Misra (1938); ten, 20 μl drops for each dilution were spotted on nutrient agar plates containing 0.3 % yeast extract, and colonies counted after 24 h incubation. Plates used for enumeration of bacteria were poured and incubated overnight at 28 °C before use, plates older than 24 h were not used.

Capillary tubes containing 10 mM phosphate buffer (pH 7.0) and casamino acids (0.1 %, wt/vol) in chemotaxis buffer, were used as controls. To ensure that the source of attractants was exudates from *A. bisporus* mycelium and not dilute CMM which had not been washed from the fungal mat, the attraction of the pseudomonads to a 1/100 dilution of the CMM culture filtrate was determined.

Chemotaxis in casing soil

An investigation of chemotaxis in casing soil was made using a modification of the method described by Bashan (1986).

A tall glass petri dish (90 mm diam x 50 mm) was unequally divided by an impermeable silicone barrier (3 mm x 50 mm x 40 mm wide). A 20 mm diam hole was cut in the centre of the barrier and a 25 mm diam, 22 μm Millipore filter, glued over the hole to prevent *A. bisporus* hyphae ramifying throughout the dish.

Casing soil, prepared by mixing 100 g of dry peat, previously passed through a 5 mm sieve, with 30 g of granulated lime (< 2 mm particle size), was placed in the Petri dish to within 10 mm of the top. The moisture content was adjusted to 175 % by weight (field capacity) and the dish and its contents were sterilized by autoclaving (120 °C, 1 h, 1.2 atm) on three successive days. The pH after autoclaving was pH 7.3. *A. bisporus* was inoculated either directly onto peat, or mushroom compost, which had been incorporated into the small section of the dish prior to autoclaving. The dishes were incubated for 1 week at 25 °C after which time 0.1 ml of bacterial suspension (10^8 cells ml^{-1}), prepared as described above, but given only a single wash in 10 mM phosphate buffer (pH 7.3), was inoculated at a central point, 50 mm from the silicone barrier.

Sampling was performed every 12 h using the sampling comb and technique described by Bashan (1986). Treatments were duplicated for each experiment.

3.2.3. ADHERENCE

Quantitative studies on attachment of bacteria to *A. bisporus* mycelium

Media, culture conditions and preparation of bacterial inoculum

Bacteria were grown on a range of different agar media to gain an indication of the effect of nutritional factors on adherence. The following media were used: KB, minimal M63 medium (Miller 1972) and M63 medium in which the concentration of carbon and nitrogen were altered: M63(50 %), 1 g l^{-1} glucose, 1 g l^{-1} $(NH_4)_2SO_4$; M63(10 %), 0.2 g l^{-1} glucose, 0.2 g l^{-1} $(NH_4)_2SO_4$; M63(C limited), 0.5 g l^{-1} glucose, 2 g l^{-1} $(NH_4)_2SO_4$; M63(N limited), 2 g l^{-1} glucose, 0.5 g l^{-1} $(NH_4)_2SO_4$. Casing soil leachate medium (CSLM) was also used and was prepared by mixing 150 of mushroom colonized casing soil with 200 ml of distilled water. This mixture was stirred for 5 min and filtered through a Whatman no.1 qualitative filter. The pH was adjusted to pH 7.3 with 1 N HCl and sterilized by autoclaving (120 °C, 15 min, 1.2 atm).

Bacteria were cultured overnight, removed from the agar medium with an inoculating loop and suspended in sterile distilled water to give an A_{610} of 0.4 (4×10^8 cells ml^{-1}). Bacteria were not washed to avoid alteration or removal of surface polymers. Bacteria grown on media of reduced nutrient status were suspended in distilled water to give an A_{610} of 0.3 (4×10^8 cells ml^{-1}). The suspension was vortex-mixed for 1 min and the cells checked microscopically for motility and aggregation before performing an experiment. If clumps of bacteria were seen the suspension was vortex-mixed for a further 10 s and rechecked microscopically.

The effect of a surfactant on the adhesion process was examined by adding 0.01 % Triton X-100 to the distilled water in which the cells were suspended (Paul & Jeffrey 1985b). The effect of Ca^{2+} ions on bacterial adhesion (the dominant cation within the casing layer environment) was examined by adding $CaCl_2$ to the distilled water to a final concentration of 5 mM.

Enumeration of bacteria

Bacterial numbers in suspensions were estimated by plating 20 μl drops on fresh (< 24 h) plates of nutrient agar supplemented with 0.3 % yeast extract (Miles & Misra 1938). Dilutions were made in PBS, in 1.5 ml Eppendorf tubes and all equipment which made contact with bacterial suspensions, such as pipette tips and tubes, were manufactured out of poly-propylene. Initial experiments revealed the inability of the bacterial isolates to adhere to this material.

Preparation of A. bisporus *cultures and attachment assay*

The method of Preece & Wong (1982) was used, but modified as follows. Petri dish cultures of *A. bisporus* growing on CMM (10 d) were flooded with sterile distilled water for 15 min to suppress aerial mycelium. Agar-mycelial plugs (10 mm diam) were aseptically removed from the margin of the cultures and placed in sterile Petri dishes. After the mycelial surface of the plug had dried (15 min), a 20 μl drop of bacterial suspension was carefully applied and spread using a pipette tip to cover the entire mycelial surface. Care was taken not to touch the mycelium with the pipette tip, or to cause the droplet to run down the side of the plug. The number of bacteria within the suspension was determined immediately after inoculation of the mycelial plug. The inoculated plugs were incubated for 30 min at 25 °C and then placed into 10 ml of PBS contained in 20 ml tubes and vortex-mixed for 1 min to dislodge any bacteria not firmly attached to the hyphal surfaces. The number of bacteria not attached to the mycelium after 30 min, or which were removed by washing, was determined by counting the number of bacteria in the 10 ml solution. The number of bacteria attached to the hyphal surfaces after 30 min was calculated by subtracting the number of bacteria initially applied to the mycelial surface of the plug, from the number of bacteria recovered after vortex-mixing. The result was expressed as a percentage of the number of bacteria initially placed on the surface of the agar-mycelial plug. The assay was replicated five times for each treatment.

Determination of bacterial hydrophobicity

Numerous methods for determining bacterial hydrophobicity have been described, but not all provide a true indication of this property (Dillon *et al.* 1986). Dillon *et al.* (1986) demonstrated the

importance of using several methods to assess the hydrophobicity. Three methods were used in this study.

Media and culture conditions

Pseudomonads were grown overnight on KB medium and CSLM.

Salt aggregation test (SAT)

The method of Lindahl *et al.* (1981) was used. A solution of 4 M $(NH_4)_2SO_4$ in 0.002 M sodium phosphate (pH 6.8 adjusted with NH_4OH) was diluted with sodium phosphate (0.002 M, pH 6.8) to give concentrations of $(NH_4)_2SO_4$ ranging from 4.0 to 2.0 M, differing by 0.2 M per solution, and from 0.2 to 0.02 M differing by 0.02 M per solution. A 25 μl volume of each $(NH_4)_2SO_4$ concentration was dispensed into a 96-well micro-titre plate and an equal volume of bacterial suspension (10^8 cells ml^{-1} in 0.002 M sodium phosphate buffer pH 6.8) was added to each filled well. The trays were then rocked for 2 min and the lowest concentration causing bacterial aggregation was the SAT value recorded.

Bacterial adherence to hydrocarbons (BATH)

A modification of the method of Rosenberg *et al.* (1980) as described by Dillon *et al.* (1986) was used. Bacteria were suspended in PUM buffer (pH 7.1) (Rosenberg *et al.* 1980) to give a final concentration of 10^8 cells ml^{-1} and 1.2 ml volumes were dispensed into round bottom 10 mm diam test tubes. Each of the hydrocarbons (*p*-xylene and *n*-hexadecane) was added to four tubes in four different volumes (0.05, 0.1, 0.15, 0.2 ml) and held at room temperature for 10 min, vortex-mixed for 2 min and stood at room temperature until the hydrocarbon separated. The absorbance of the aqueous phase was measured with a 'MSE Spectro-plus' spectrophotometer using a wave length of 400 nm and 1 cm cuvettes. The results were recorded as the percentage absorbance of the aqueous phase relative to the initial absorbance of the bacterial suspension.

Adherence to polystyrene

The procedure described by Rosenberg (1981) was used. A polystyrene Petri dish lid was pressed firmly onto bacterial colonies growing on the surface of agar plates. The lid was

removed and washed for 2 min under rapidly running water, fixed in methanol and stained with crystal violet. A positive result was recorded if greater than 50 % of the colony remained attached to the polystyrene lid.

Electron and light microscopy

Methods employed for processing material for electron microscopy followed standard procedures (see Hayat 1970, 1972).

Media, culture conditions and preparation of bacterial inoculum

Bacterial isolates were grown overnight on KB plates and suspended in sterile distilled water to a concentration of 1×10^8 cells ml^{-1}. Cells were checked microscopically for motility before performing an experiment.

Preparation of A. bisporus *cultures*

A. bisporus cultures were grown for 10 d on CMM and flooded with 10 ml of bacterial suspension and re incubated at 25 °C. Plugs of agar and mycelium, 5 mm diam, were removed from the margin of the colony with a sterile cork borer 30 min, 3 h, 24 h and 72 h after inoculation. The agar-mycelial plugs were washed twice in sterile distilled water, for 5 min on each occasion, before fixing in glutaraldehyde (see below). Some plugs removed from the *A. bisporus* culture 24 h after inoculation were placed in 5 ml of sterile distilled water, where they were left for a further 48 h before fixation. Some plugs incubated for extended periods of time were plated onto CMM to check for fungal growth. All treatments were examined by scanning electron microscopy, but only the 30 min, 3 h and 24 h treatments were examined by transmission electron microscopy.

Scanning electron microscopy (SEM)

Fixation Mycelial mats on agar were fixed by immersion in 3 % glutaraldehyde in 0.075 M phosphate buffer (pH 7.2) for 3 h at 20 °C. During the fixation process the specimens were briefly evacuated to remove trapped air. After fixation the agar-mycelial plugs were washed in 0.075 M phosphate buffer (pH 7.2) with four changes of 30 min duration.

Dehydration Specimens were dehydrated in a series of ethanol-distilled water mixtures (30, 50, 70, 80, 90, 95, 100 % ethanol) for 2 h in each dilution and put through a series of amylacetate-ethanol mixtures (25, 50, 75, 100 % amylacetate) for 4 h in each dilution.

Tissues were critical point dried, mounted on stubs and gold coated for 3 min in a 'Polaron' diode splutter device (E5000) and examined with a 'Cambridge stereoscan 250 Mk 2' scanning electron microscope. Micrographs were taken on 'Ilford FP4' 35 mm (developed in 'Microphen' developer rated at 200 ASA).

Transmission electron microscope (TEM)

Fixation Mycelial mats on agar were fixed as for SEM and washed in 0.075 M phosphate buffer (pH 7.2) with three changes of 10 min duration. Post fixation was for 3 h in 1 % osmium tetroxide in 0.075 M phosphate buffer followed by three changes, each of 20 min duration, in the same buffer.

Dehydration Tissues were dehydrated in an acetone-distilled water series with 15 min in 20, 40, 60 and 80 % acetone; two 30 min changes in 100 % acetone.

Infiltration and embedding Specimens were infiltrated overnight with 25 % Spurr's (Spurr 1969) resin in acetone followed by a 3 h period in 75 % Spurr's resin in acetone. Embedding was in 100 % Spurr's resin polymerized for 24 h at 70 °C.

Ultramicrotomy Specimens were cut out of resin blocks and glued to araldite stubs and hand trimmed to form blocks (Fineran 1971). Sections, 4 μm thick, were cut on a 'L. K. B. Pyramitome' (type 11800) equipped with glass knives. Ultrathin sections were cut on an 'L. K. B. Ultrotome III' (type 4800A) using diamond knives. Sections were collected on uncoated 300 mesh and coated 100 mesh copper grids.

Staining Grids were stained routinely with uranyl acetate (Watson 1958). Selected grids were stained so as to enable detection of polysaccharide material. The Thiery reaction (Thiery 1967) with appropriate controls was employed for the detection of periodate-sensitive polysaccharides.

Specimens were examined in a JEM-1200EX electron microscope at an accelerating voltage of 80 Kv and micrographs were taken on Agfa Ortho 35 mm film and developed in Rodinol and on Kodak Electron Microscope film (4489) and developed in Kodak D-19 developer.

Light microscopy

Specimens were examined directly and usually without staining using a Reichert microscope equipped with interference optics and an Olympus BH2 microscope equipped with bright field optics. Bacterial capsules were detected by negative staining using Indian ink. Micrographs were taken on Ilford Pan F 120 or Ilford FP4 35 mm film and developed in Rodinol.

3.2.4. STATISTICAL ANALYSIS

Statistical analysis was performed using standard ANOVA techniques. Comparisons between means were made using Fisher's protected least significant difference test (L.S.D.).

3.3. RESULTS

3.3.1. CHEMOTAXIS

The results of the chemotaxis assays are shown in Table 3.2. Each isolate demonstrated a significant, positive taxis toward the mycelial exudates ($P < 0.05$). Heat treating the exudate had little effect on its ability to attract the smooth colonial form of *P. putida* PMS118, but significantly decreased its ability to attract the smooth colonial form of *P. tolaasii* PMS117. The rough colonial forms of *P. putida* PMS118 and *P. tolaasii* PMS117 both displayed an increased taxis toward the heat treated exudate, when compared with the untreated exudate.

Table 3.2. Chemotaxis of smooth and rough colonial forms of *P. putida* PMS118 and *P. tolaasii* PMS117 toward untreated, heated, and dialyzed exudates of *A. bisporus* mycelium, phosphate buffer, and casamino acids.

	Log number of bacteria (cells ml^{-1})			
Attractant	**PMS118S[1]**	**PMS118R**	**PMS117S**	**PMS117R**
Buffer (control)	3.69^{a}	3.66^{a}	3.76^{a}	3.79^{a}
Untreated exudate	4.50^{b}	4.43^{b}	4.52^{b}	4.57^{b}
Heated exudate	4.45^{b}	4.59^{c}	4.10^{c}	4.68^{c}
Dialyzed exudate	3.80^{a}	3.23^{a}	4.16^{c}	3.87^{a}
Casamino acids	4.18^{c}	4.14^{d}	4.24^{c}	4.26^{d}

[1]S, smooth colonial form; R, rough colonial form. Data are means of four experiments; within a column there is no significant difference ($P < 0.05$) between data with the same letter.

The response of the smooth colonial form of *P. tolaasii* PMS117 to the dialyzed exudate was significantly greater than the control, but the rough colonial form of *P. tolaasii* PMS117 and both colonial forms of *P. putida* PMS118 displayed no significant taxis toward this source of attractants.

Casamino acids attracted all isolates, but to a lesser extent than the untreated mycelial exudate. Attraction of the pseudomonads to the dilute culture filtrate was not significantly greater than the buffer control (data not shown) which confirmed that the source of attractants was *A. bisporus* mycelium and not dilute culture filtrate.

Migration of *P. putida* PMS118S and *P. tolaasii* PMS117S in casing soil toward the section containing mushroom mycelium was observed, but only occurred when *A. bisporus* was growing vigorously which required incorporation of mushroom compost into the small section of the Petri dish. Consequently, it was not possible to differentiate chemotaxis toward diffusates from the nutritional substrate, from chemotaxis toward the mycelium, nevertheless, migration of the pseudomonads toward a source of nutrients in casing soil was demonstrated. *P. putida* PMS118S reached the silicone barrier in 48 h, while *P. tolaasii* PMS117S took 72 h to cover the same distance.

3.3.2. ADHERENCE

Quantitative studies on adherence of *P. putida* PMS118 and *P. tolaasii* PMS117 to *A. bisporus* mycelium

The adherence of smooth and rough colonial forms of *P. putida* PMS118 to *A. bisporus* mycelium is shown in Fig. 3.1. A two-way ANOVA revealed no significant difference between the ability of the smooth and rough forms to adhere to the mycelium, but the media on which the cells were grown, prior to performing the attachment assay, had a significant affect on bacterial adherence ($P < 0.001$). The interaction between the different forms and media on which they were grown was also significant ($P < 0.001$).

Comparisons between means revealed a number of significant differences. When the smooth colonial form of *P. putida* PMS118 was grown on either, KB, or M63, the percentage attachment was significantly less than the rough form ($P < 0.05$). Reducing the amount of carbon and nitrogen in M63 did not affect the ability of either form to adhere to the mycelium, but when cultured on C-limited M63, the smooth colonial form attached in significantly greater numbers than it did when grown on KB, M63 or N-limited M63 ($P < 0.001$). Growth on CSLM caused both colonial forms to attach to the mycelium in large numbers and the attachment of the

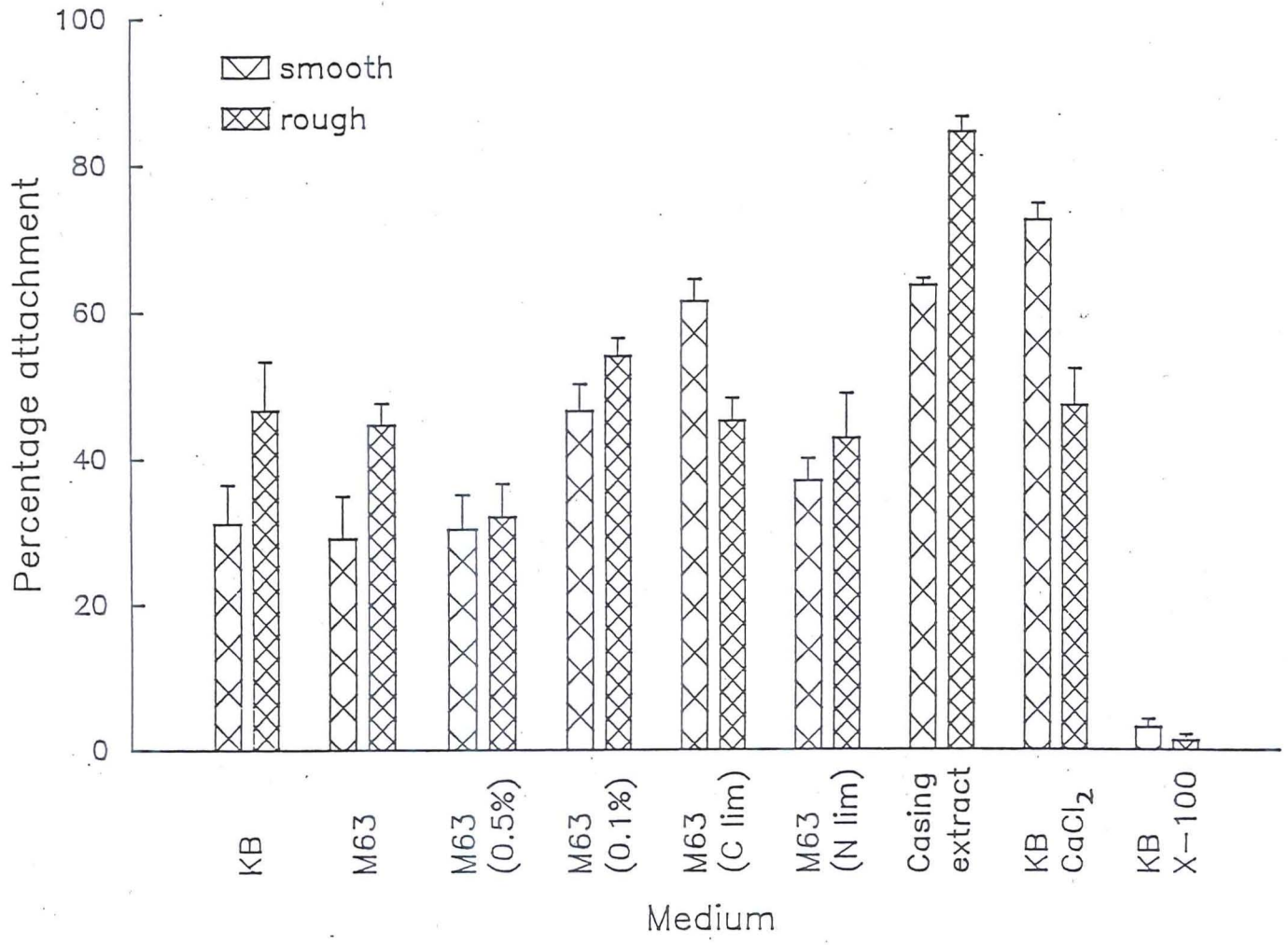


Fig. 3.1. The effect of culture conditions on attachment of smooth and rough colonial forms of *P. putida* PMS118 to *A. bisporus* mycelium. Data are means and S.E. of five replicates. See text (section 3.2.3) for details of media.

rough form was significantly greater than on any other medium ($P < 0.001$). When the attachment assay was conducted in the presence of 5 mM $CaCl_2$ there was a significant increase in adherence of the smooth colonial form to mycelium ($P < 0.001$) when compared to adherence of this form after growth on KB or M63. $CaCl_2$ did not increase the percentage attachment of the rough colonial form when compared to the adherence of this form after growth on these media. Triton X-100 significantly decreased the attachment of both colonial forms ($P < 0.001$).

The adherence of smooth and rough colonial forms of *P. tolaasii* PMS117 to *A. bisporus* mycelium is illustrated in Fig. 3.2. There was a significant difference between the ability of the two colonial forms to adhere to mycelium ($P < 0.001$) and the effect of different media on adherence was also significant. The interaction between these two factors was significant ($P < 0.001$).

The smooth colonial form consistently attached to the mycelium in greater numbers than the rough colonial form and attachment of *P. tolaasii* PMS117S was greatest when cultured on N-limited M63 and CSLM. Compared with these two media, the percentage attachment of the smooth colonial form decreased significantly when the attachment assay was performed in the presence of $CaCl_2$, but the number of bacteria which adhered to the mycelium was comparable to the number of bacteria which attached after culture on KB or M63. On M63 containing 10 % less carbon and nitrogen (0.1% M63), attachment of the smooth colonial form was significantly reduced ($P < 0.05$), compared to the attachment of this form after growth on KB and M63. The percentage attachment of the rough colonial form to the mycelium was greatest when cultured on M63 containing 10 % less carbon and nitrogen. Conducting the attachment assay in the presence of $CaCl_2$, or growing the rough form on CSLM, had little effect on its ability to adhere to the mycelium.

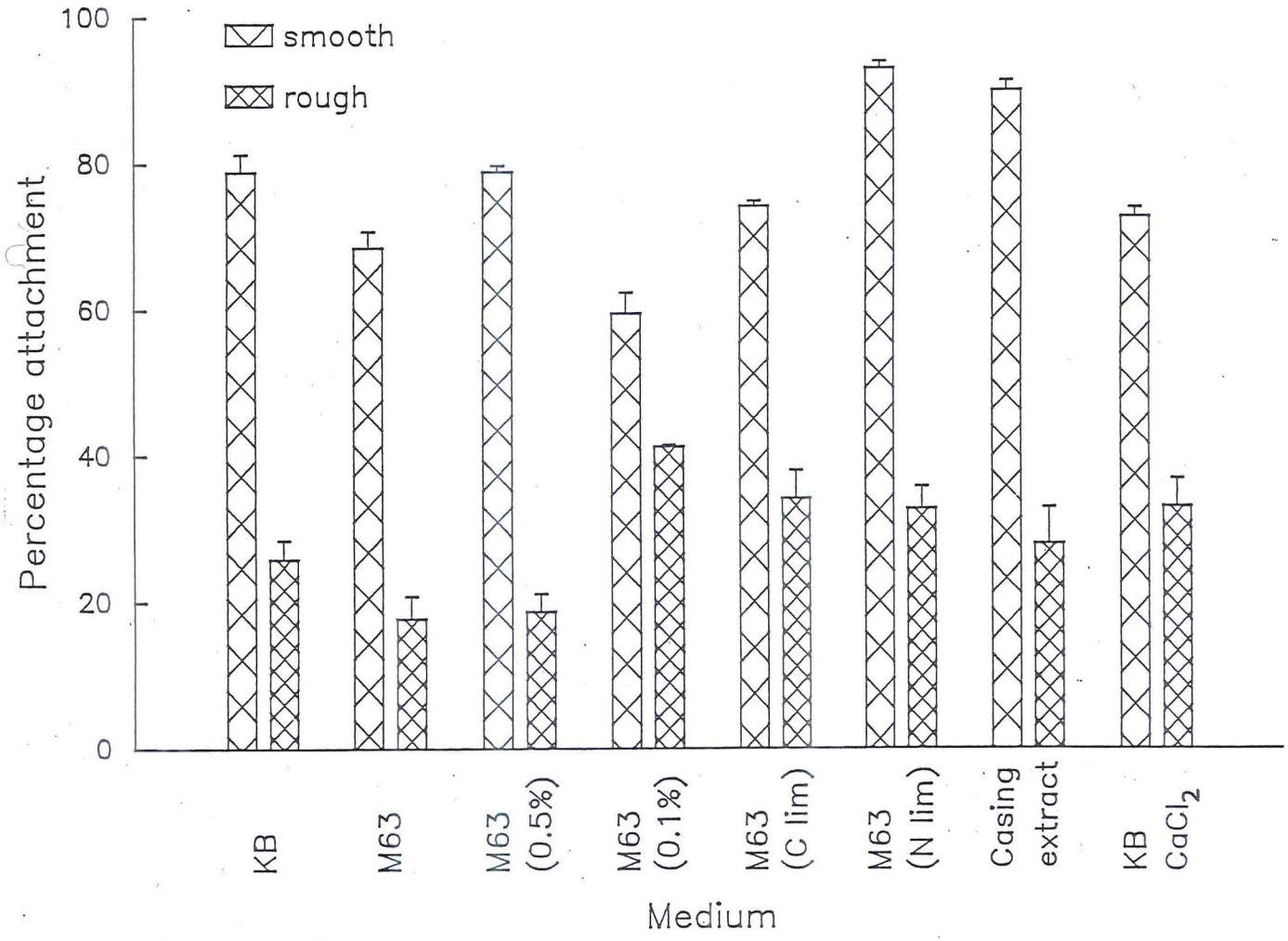


Fig. 3.2. The effect of culture conditions on attachment of smooth and rough colonial forms of *P. tolaasii* PMS117 to *A. bisporus* mycelium. Data are means and S.E. of five replicates. See text (section 3.2.3) for details of media.

Determination of hydrophobicity of *P. putida* PMS118 and *P. tolaasii* PMS117

Salt aggregation test (SAT)

The results from the SAT assay are shown in Table 3.3. The SAT assay indicated that both the smooth and rough colonial forms of *P. tolaasii* PMS117 were moderately hydrophobic and the hydrophobicity of the smooth form increased after growth on CSLM. An end point was not produced for KB cultured *P. putida* PMS118 colonial forms, but aggregation did occur in 4.0 M ammonium sulphate after the cells were grown on CSLM indicating that both *P. putida* PMS118 colonial forms were relatively hydrophilic.

Table 3.3. Hydrophobicity of smooth and rough forms of *P. putida* PMS118 and *P. tolaasii* PMS117 after growth on KB and CSLM as determined by the salt aggregation test (SAT).

	Medium	
Bacterial isolate	**KB**	**CSLM**
P. putida PMS118S[2]	> 4.0[1]	4.0
P. putida PMS118R	> 4.0	4.0
P. tolaasii PMS117S	2.0	1.6
P. tolaasii PMS117R	2.0	2.0

[1]Lowest molarity giving bacterial aggregation
[2]S, smooth colonial form; R, rough colonial form

Examination of the cells after growth on CSLM revealed cells approximately half the size (0.5 - 0.75 μm) of those grown on KB. Indian ink staining showed the presence of an amorphous capsule on KB cultured cells, but not on CSLM grown cells.

Bacterial adherence to hydrocarbons (BATH)

Figs 3.3 and 3.4 show the adherence of smooth and rough colonial forms of *P. putida* PMS118 and *P. tolaasii* PMS117 to xylene and hexadecane. Adherence of the isolates to xylene after growth on KB indicated that both colonial forms of *P. putida* PMS118 were weakly hydrophobic and the two forms of *P. tolaasii* PMS117 were slightly hydrophobic. Hexadecane revealed similar results, although the rough colonial form of *P. putida* PMS118 displayed slightly greater

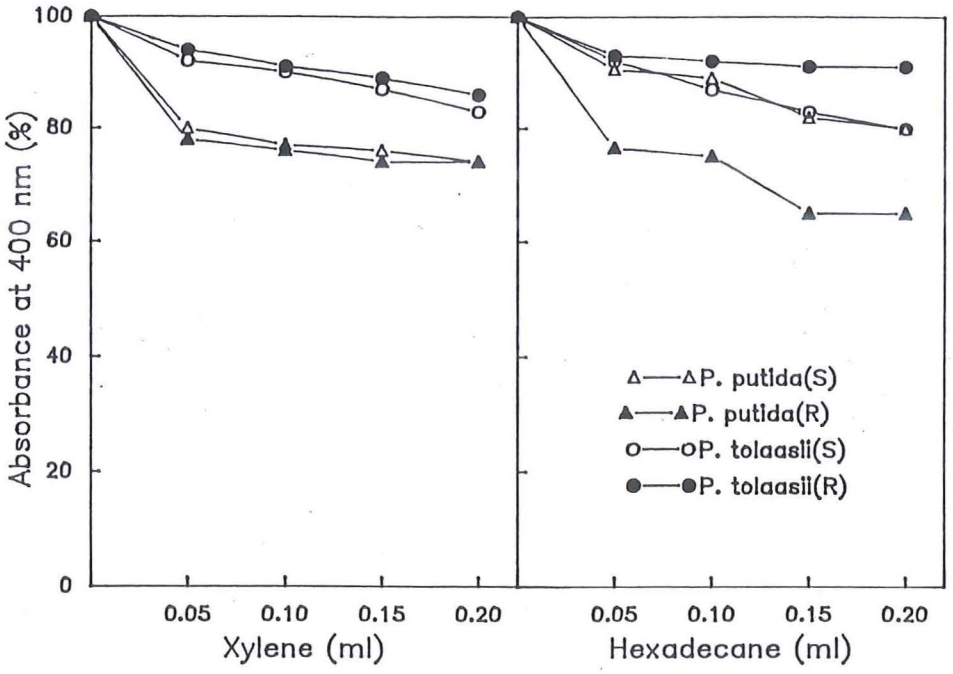


Fig. 3.3. Adherence of smooth and rough colonial forms of *P. putida* PMS118 and *P. tolaasii* PMS117 to varying volumes of *p*-xylene and *n*-hexadecane after growth on KB medium in the BATH assay. Data are means of duplicates

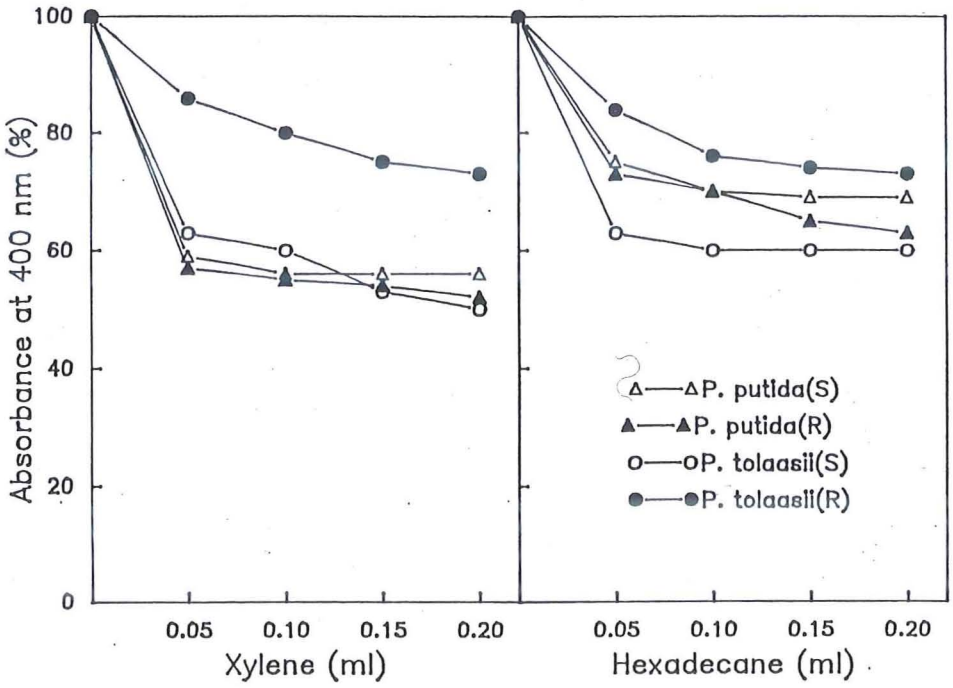


Fig. 3.4. Adherence of smooth and rough colonial forms of *P. putida* PMS118 and *P. tolaasii* PMS117 to varying volumes of *p*-xylene and *n*-hexadecane after growth on CSLM medium in the BATH assay. Data are means of duplicates.

hydrophobicity than the smooth colonial form. The hydrophobicity of all isolates increased after growth on CSLM and moderate hydrophobicity was indicated for all isolates except the rough colonial form of *P. tolaasii* PMS117.

Bacterial adherence to polystyrene

Both colonial forms of *P. putida* PMS118 and *P. tolaasii* PMS117 scored a weak positive result for this test.

Microscopic studies of adherence

Scanning electron microscopy (SEM)

Figs 3.5 - 3.13 show bacteria attached to *A. bisporus* mycelium after 30 min. Cells of both smooth and rough colonial forms of *P. putida* PMS118 adhered to the mycelium in large numbers and in most instances the bacteria lay flat against the hyphae (Figs 3.5 and 3.6), although some were seen attached by one end only. *P. tolaasii* PMS117S cells and *P. aeruginosa* OT11 cells also attached to the mycelium in large numbers and were often orientated perpendicular to hyphal surfaces (Figs 3.8 and 3.10). Few cells of the rough colonial form of *P. tolaasii* PMS117, or *P. syringae* PDDCC7607, were seen attached to the mycelial surfaces (Figs 3.7 and 3.9). Attachment was not uniform over all hyphae and young hyphae tended to have more attached bacteria; bacteria were sometimes observed attached in large numbers to a single hypha, while adjacent hyphae were devoid of bacteria. These observations were confirmed by light microscopy

Fibrillar rods were frequently associated with the adsorbed bacteria (Fig. 3.13) and appeared to joined the bacteria both to the mycelial surfaces and to each other. These rod like structures did not resemble flagella, or fimbrae.

Differences in the patterns of attachment, especially of the smooth and rough colonial forms of *P. putida* PMS118, became evident after the bacteria were left for longer than 30 min in the presence of the mycelium. Examination of the mycelial plugs after 3 h and 24 h revealed little change, although the number of *P. putida* PMS118R cells attached appeared to decline. After 72 h in the presence of the mycelium (the last 48 h in distilled water), very few *P. putida* PMS118S

cells were seen attached to the mycelium and those present were confined to small micro-colonies (Fig. 3.14a). In contrast, a large number of *P. putida* PMS118R cells were attached and were evenly distributed over the mycelial surface. These cells were orientated perpendicular to the mycelial surface (Fig. 3.14b). The number of cells of the smooth colonial form of *P. tolaasii* PMS117 which remained in contact with the mycelium after this treatment declined, but distinct micro-colonies were not observed (Fig. 3.16). Few cells of the rough form of this species were seen in contact with the mycelium. Similar results were observed when this material was examined by light microscopy, without prior fixation or staining (Figs 3.19a and 3.19b).

Examination of the mycelial plugs 72 h after inoculation of CMM cultures with bacteria revealed a large number of *P. putida* PMS118S and *P. tolaasii* PMS117S cells attached to the mycelium and copious production of slime was evident (Figs 3.15a, 3.16 and 3.17). There was also evidence of slime production by the rough colonial form of *P. putida* PMS118 (Fig. 3.15b), but there appeared to be a decrease in the number of attached cells. Few cells of the rough colonial form *P. tolaasii* PMS117 were observed in contact with the mycelium (Fig. 3.18).

Mycelial plugs removed from the 'broth' after 3 d grew when plated on CMM.

Transmission electron microscopy (TEM)

TEM enabled a detailed examination of the means by which the bacteria attached to the mycelial surface. Figs 3.23a - 3.24b show the sequence of events leading to attachment of the smooth colonial form of *P. putida* PMS118. Initial attachment appeared to occur after contact was made between the uneven surface of the bacterium and the hyphae. An amorphous material was then produced and served to anchor the bacterium firmly to the hyphal surface. TCH staining revealed this material to be polysaccharide and it formed a continuum between the bacterium and surface of the fungus, firmly anchoring the bacterium (Figs 3.25 - 3.27).

The sequence of events leading to attachment of the rough colonial form of *P. putida* PMS118 is shown in Figs 3.28 - 3.33. The surface of this organism was considerably more uneven and fibrillar rods, frequently longer than 100 nm, protruded from the surface. These appeared to facilitate the initial stage of the adhesion process (Fig. 3.29) and TCH staining indicated that they were polysaccharide in nature (Figs 3.31a and 3.31b). Fibrillar rods were also

evident in the 24 h material, but were more numerous and longer (up to 300 nm) than those seen after 3 h. (Fig. 3.33)

Short fibrillar rods were also seen protruding from the surface of *P. tolaasii* PMS117S cells and these also appeared to have a role in the adhesion process. Evidence of polysaccharide deposition between the attached cell and the hyphal surface was frequently seen in 24 h specimens (Figs 3.34 and 3.35a). No *P. tolaasii* PMS117R cells were observed by TEM, despite examination of a number sections. This was indicative of the low number of cells which attached to the mycelium.

Light microscopy

Light microscopy revealed the presence of intra-hyphal bacteria and these are shown in Figs 3.20 and 3.21. Evidence of hyphal penetration was only ever observed on mycelial plugs which had been inoculated with the smooth colonial form of *P. putida* PMS118. The production of large capsules by both the smooth and rough colonial forms of *P. putida* PMS118 was also seen with the light microscope (Fig. 3.22). After growing in the presence of the mycelium for 48 h the bacteria frequently developed large capsules and attached perpendicular to the mycelial surface. Often the capsulated bacteria completely surrounded the mycelium forming a thick sheath. The attached bacteria continued to grow and divide resulting in the formation of chains of bacteria, up to eight cells in length. These remained orientated perpendicular to the mycelial surface, leaving only the first cell attached. Capsules were never detected when these bacteria were cultured on KB in the absence of the mycelium.

Fresh material taken from the casing layer of commercial farms was also examined by light microscope, but the nature of this material made photography difficult. Nevertheless, many bacteria were observed attached to hyphae and were frequently seen alongside calcium oxalate crystals.

Fig. 3.5. *P. putida* PMS118S attached to *A. bisporus* mycelium (30 min). Fibrillar rods can be seen between bacteria and between bacteria and hyphae. (Bar = 10 μm)

Fig. 3.6. *P. putida* PMS118R attached to *A. bisporus* mycelium (30 min). Fibrillar rods facilitating attachment of the bacteria can be seen. (Bar = 10 μm)

Fig. 3.7. *P. syringae* attached to *A. bisporus* mycelium (30 min). (Bar = 10 μm)

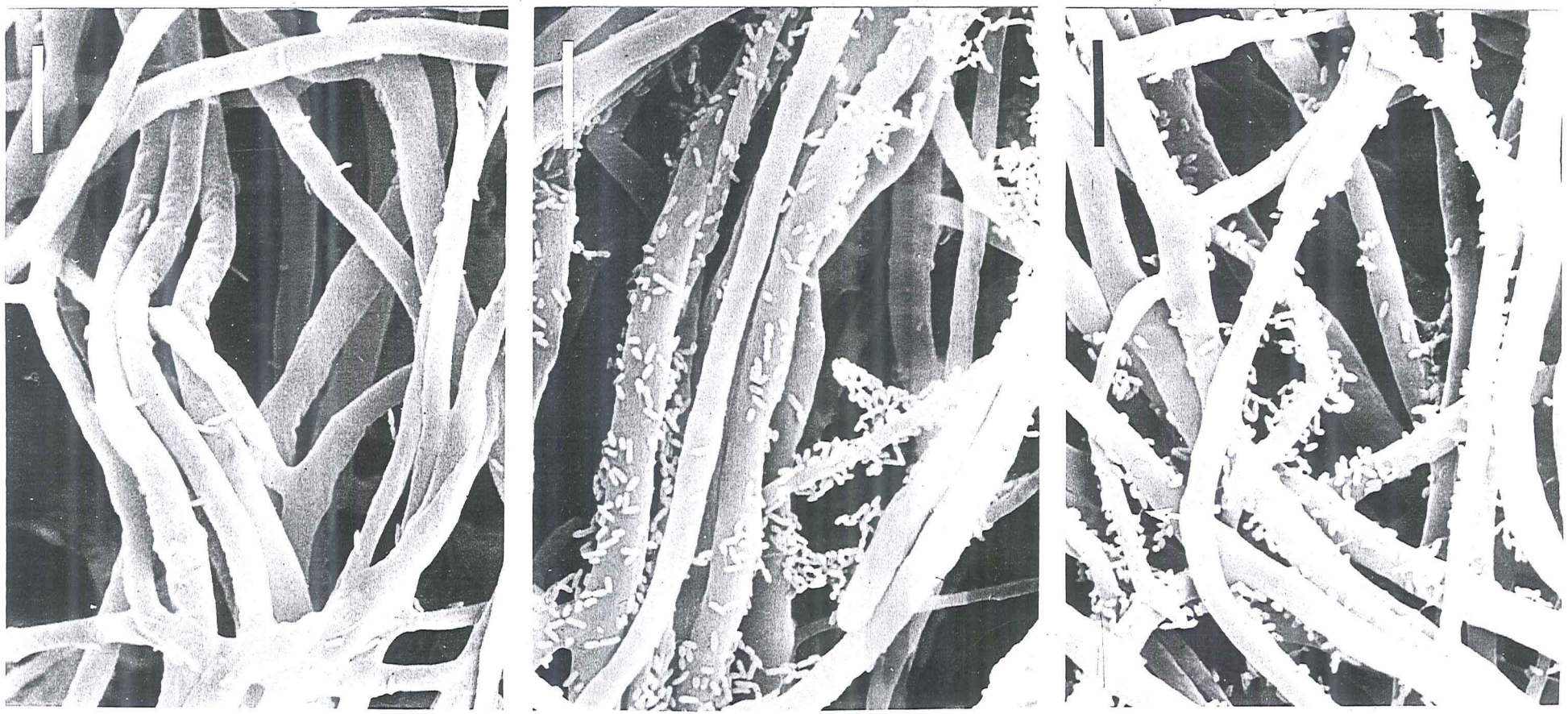


Fig. 3.8. *P. tolaasii* PMS117S attached to *A. bisporus* mycellum (30 min). Note cells attached perpendicular to hyphal surfaces. (Bar = 10 μm)

Fig. 3.9. *P. tolaasii* PMS117R cells attached to *A. bisporus* mycelium (30 min). (Bar = 10 μm)

Fig. 3.10. *P. aeruginosa* attached to *A. bisporus* mycelium (30 min). Note cells attached perpendicular to hyphal surfaces and presence of fibrillar rods. (Bar = 10 μm)

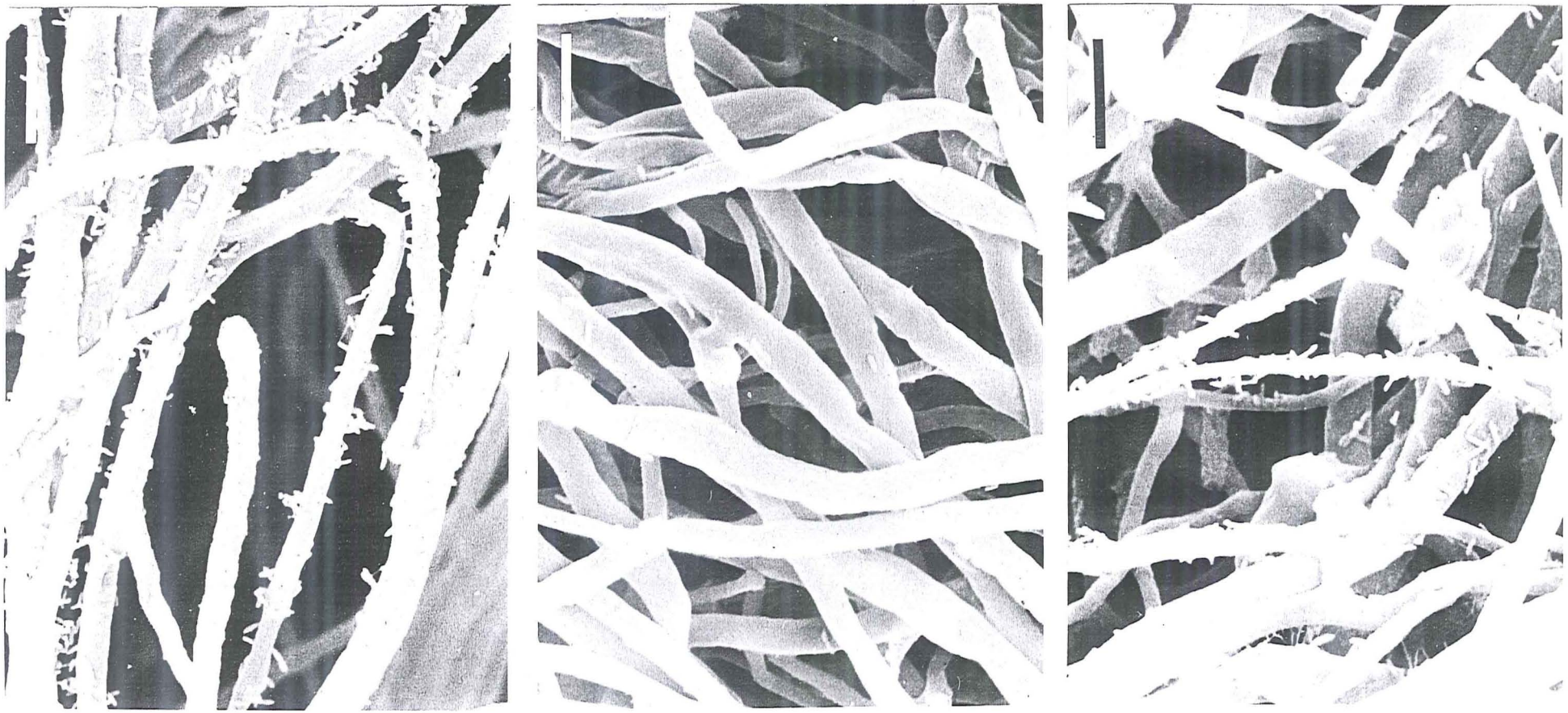
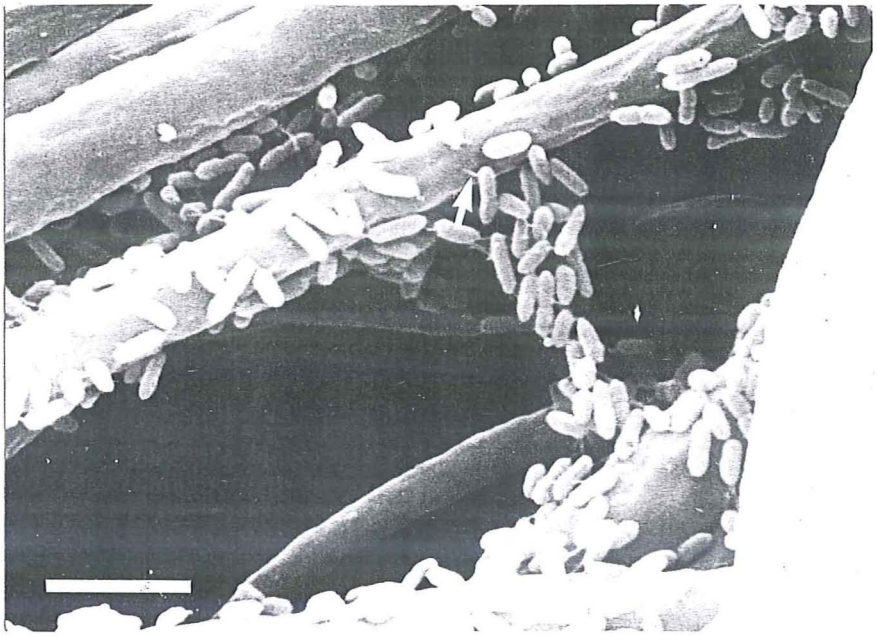
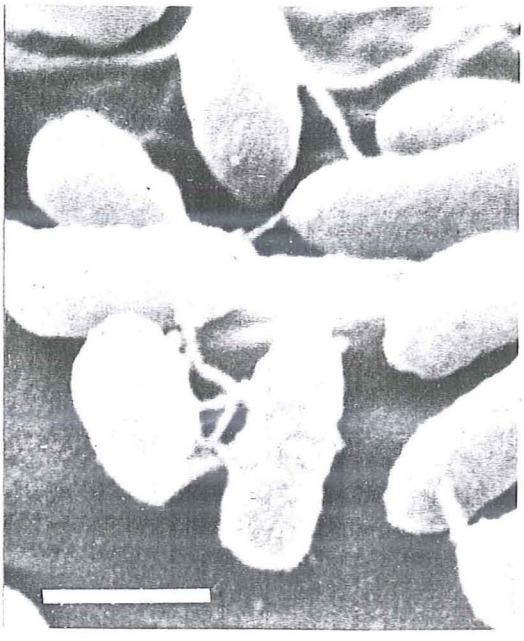


Fig. 3.11. Fibrillar rods associated with the attachment of *P. putida* PMS118S to *A. bisporus* mycelium (30 min). (Bar = 5 μm)

Fig. 3.12. Fibrillar rods associated with the attachment of *P. putida* PMS118R to *A. bisporus* mycelium (30 min). (Bar = 5 μm)

Fig. 3.13. Detail of fibrillar rods; (a) associated with *P. putida* PMS118S; (b) associated with *P. putida* PMS118R. The surface of the smooth form is more uneven than the surface of the rough (30 min). (Bar = 1 μm)





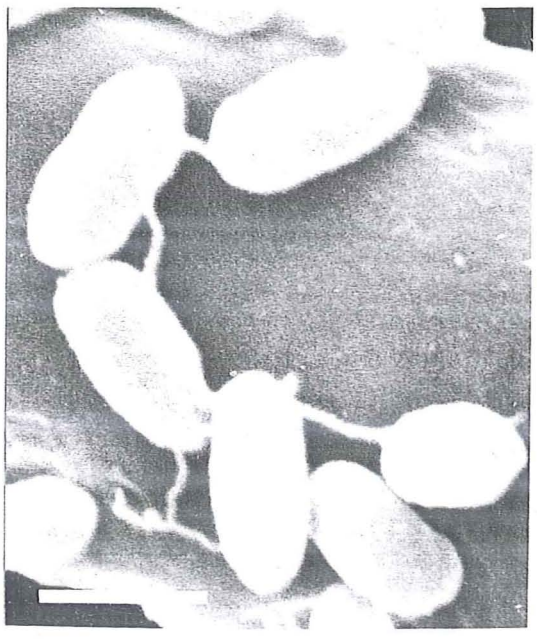


Fig. 3.14a. Micro-colonies of *P. putida* PMS118S attached to *A. bisporus* mycelium (24 h on CMM and 48 h in distilled water). (Bar = 5 μm)

Fig. 3.14b. *P. putida* PMS118R attached perpendicular to *A. bisporus* hyphal surfaces (24 h on CMM and 48 h in distilled water). (Bar = 5 μm)

Fig. 3.15a. *P. putida* PMS118S attached to *A. bisporus* mycelium (72 h; nutrient rich conditions). Large amounts of slime are present and can be seen anchoring the bacteria to the hyphae. (Bar = 5 μm)

Fig. 3.15b. *P. putida* PMS118R attached to *A. bisporus* mycelium (72 h; nutrient rich conditions). (Bar = 5 μm)

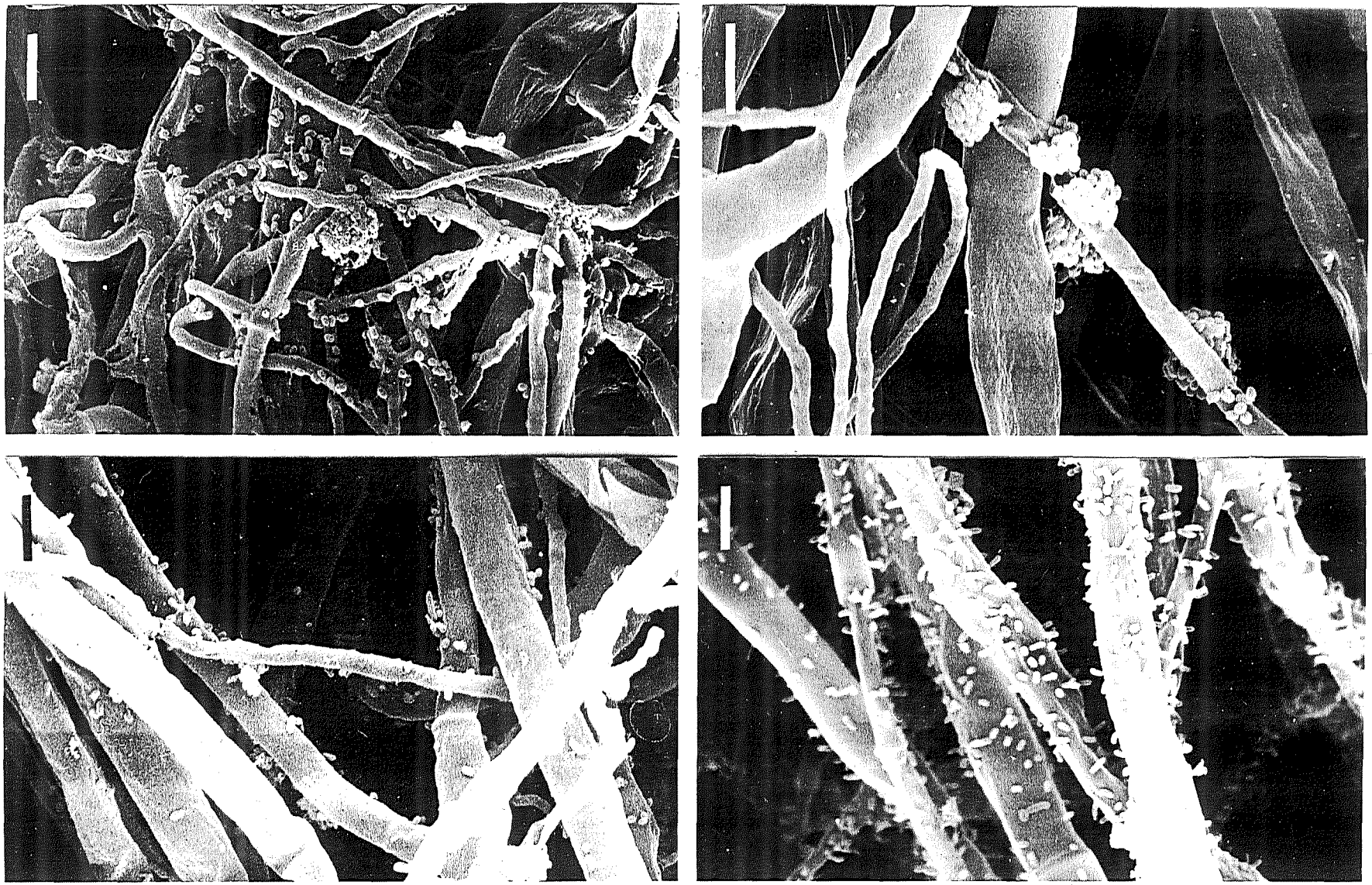
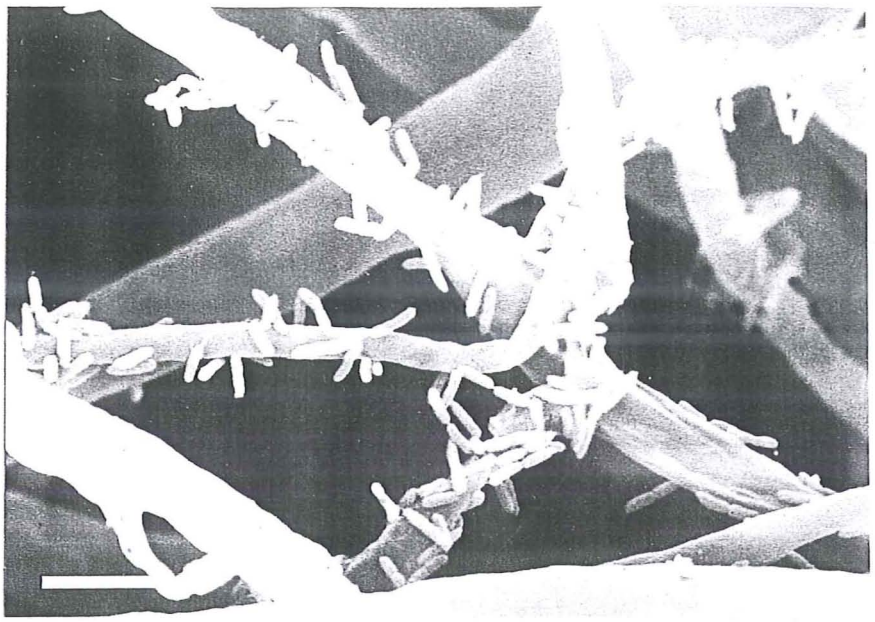


Fig. 3.16. *P. tolaasii* PMS117S attached to *A. bisporus* mycelium (30 min). (Bar = 5 μm)

Fig. 3.17. *P. tolaasii* PMS117S attached to *A. bisporus* mycelium (24 h on CMM and 48 h in distilled water). (Bar = 1 μm)

Fig. 3.18. *P. tolaasii* PMS117R attached to *A. bisporus* mycelium (24 h on CMM and 48 h in distilled water). (Bar = 10 μm)



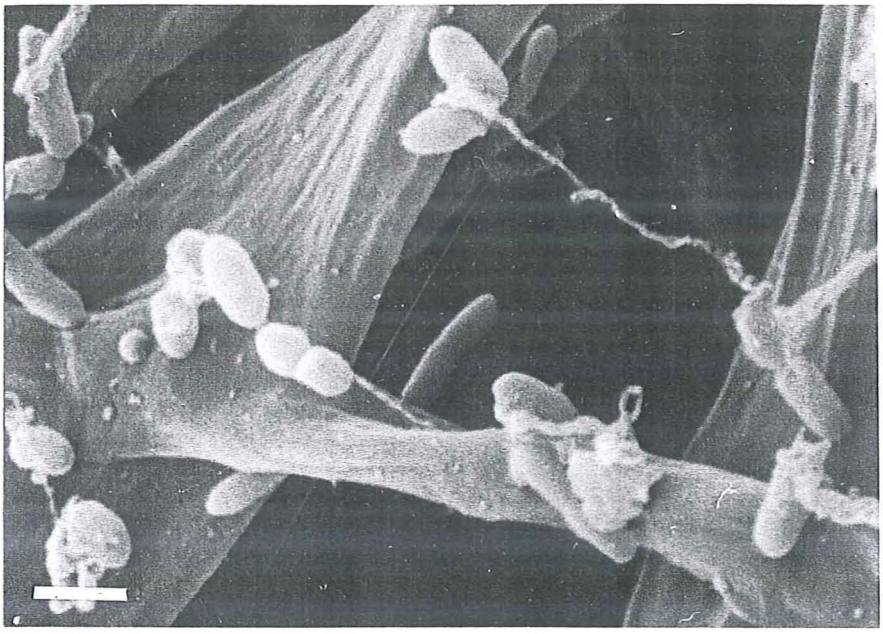


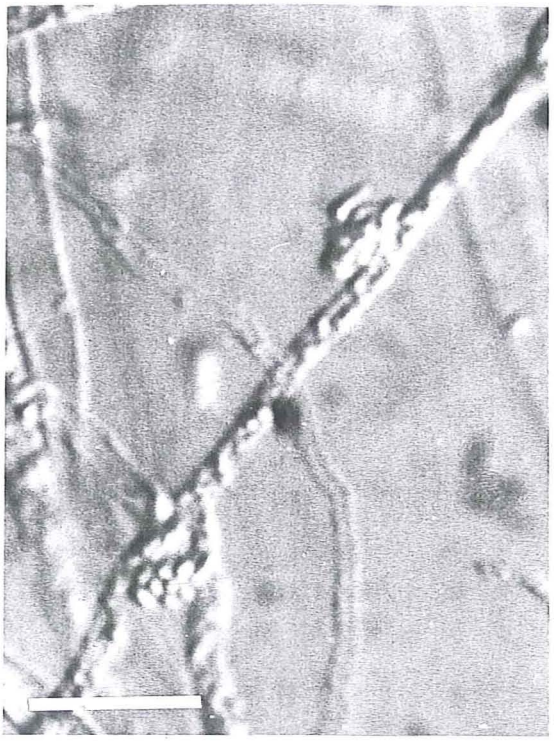
Fig. 3.19a. Interference (oil immersion) micrograph of micro-colonies of *P. putida* PMS118S attached to *A. bisporus* mycelium (24 h on CMM and 48 h in distilled water). (Bar = 10 μm)

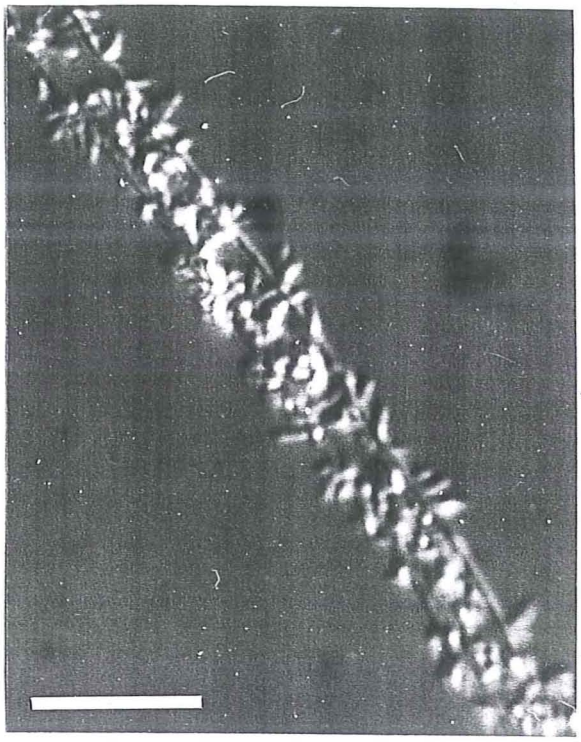
Fig. 3.19b. Interference (oil immersion) micrograph of *P. putida* PMS118R attached perpendicular to *A. bisporus* hyphal surfaces (24 h on CMM and 48 h in distilled water). (Bar = 10 μm)

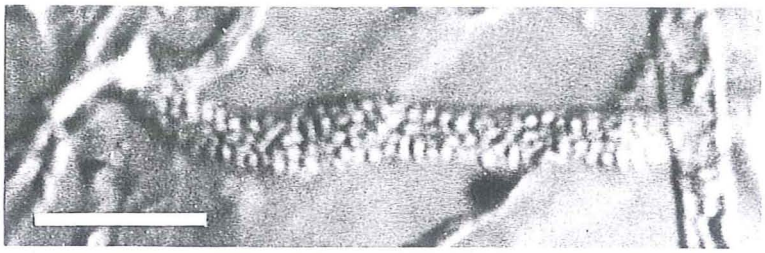
Fig. 3.20. Interference (oil immersion) micrograph of *P. putida* PMS118S within an *A. bisporus* hyphal compartment (24 h on CMM and 48 h in distilled water). (Bar = 10 μm)

Fig. 3.21. Interference (oil immersion) micrograph of *P. putida* PMS118S within an *A. bisporus* hyphal tip compartment (24 h on CMM and 48 h in distilled water). (Bar = 10 μm)

Fig. 3.22. Light micrograph of capsulated *P. putida* PMS118R surrounding *A. bisporus* mycelium. Mycelium is indicated with an arrow. (Bar = 10 μm)







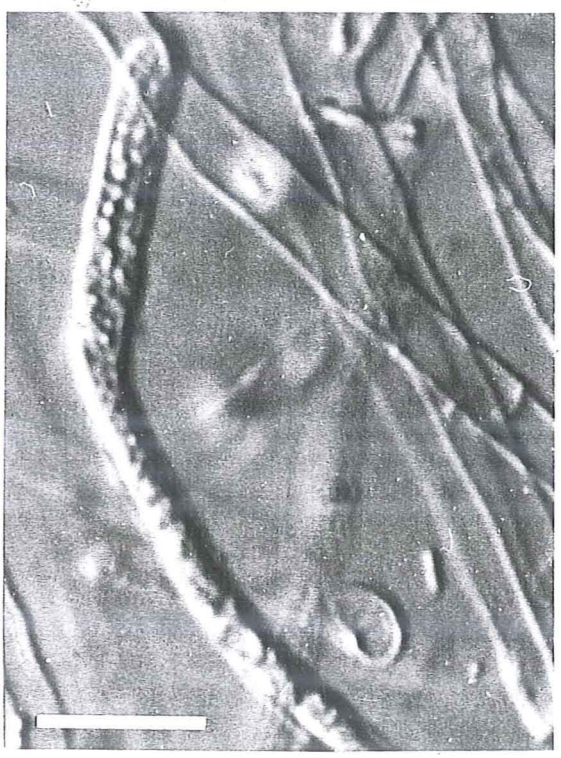
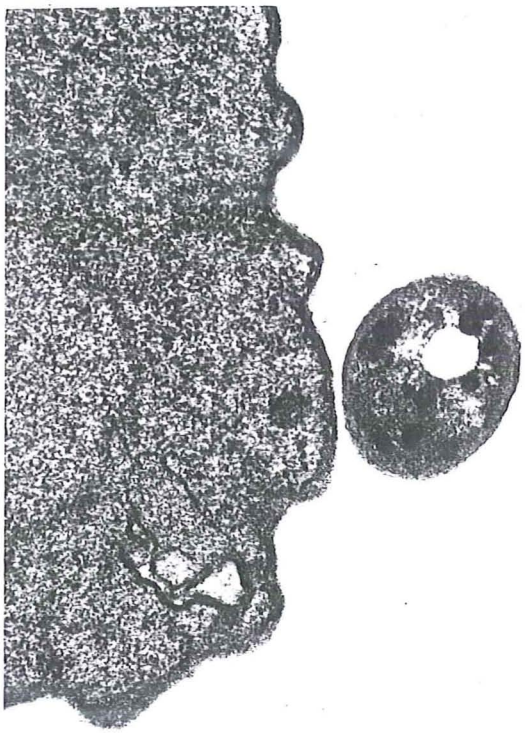


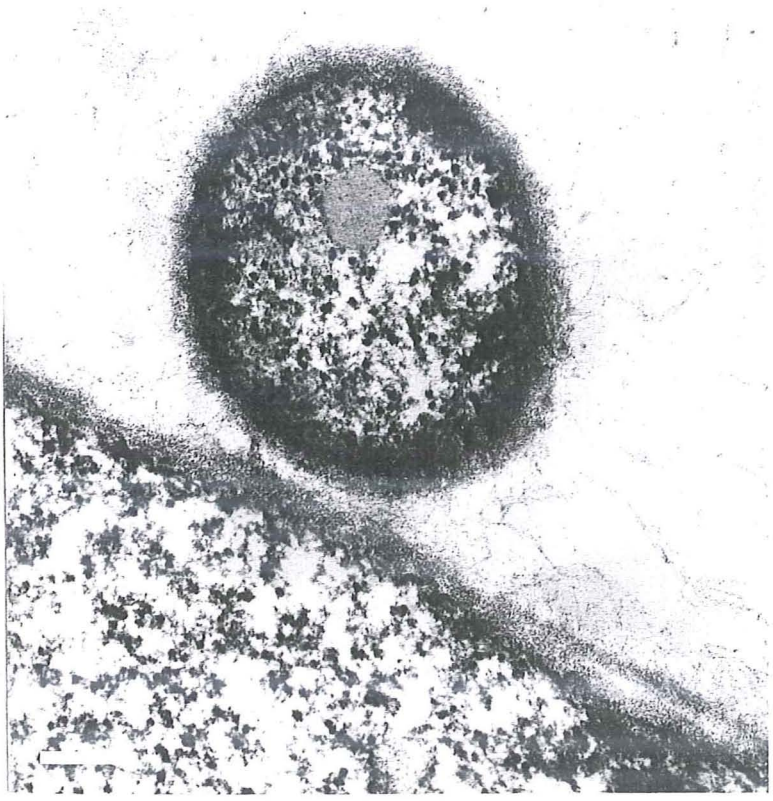
Fig. 3.23a. *P. putida* PMS118S attached to *A. bisporus* hypha (30 min). (Bar = 200 nm)

Fig. 3.23b. *P. putida* PMS118S attached to *A. bisporus* hypha (3 h). Note build up of anchoring material between bacteria and hyphae. (Bar = 100 nm)

Fig. 3.24a. *P. putida* PMS118S attached to *A. bisporus* hypha (24 h). Note build up of anchoring material between bacteria and hyphae. (Bar = 200 nm)

Fig. 3.24b. High power micrograph of Fig. 3.24a showing anchoring matrix. (Bar = 100 nm)





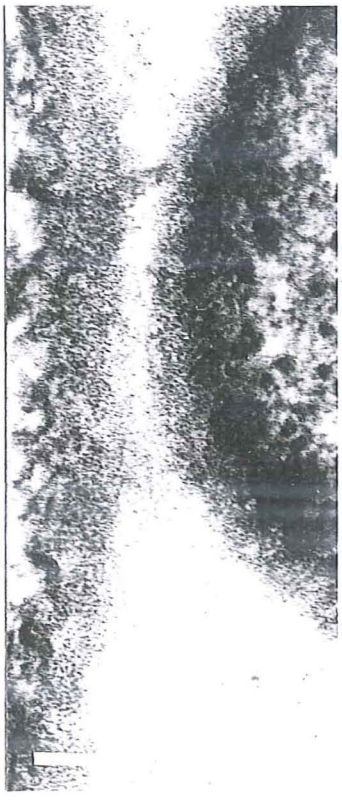
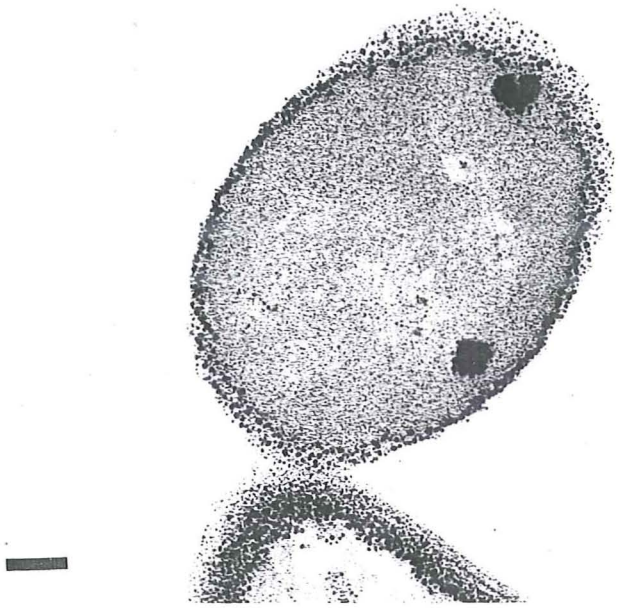


Fig. 3.25. *P. putida* PMS118S attached to *A. bisporus* hypha (3 h) (TCH stained). A layer of polysaccharide material surrounds the bacterium and is continuous with the polysaccharide layer of the fungus. (Bar = 100 nm)

Fig. 3.26. As for Fig. 3.25.

Fig. 3.27. Control for TCH stain. *P. putida* PMS118S attached to *A. bisporus* hypha (3 h). Note unstained layer surrounding the bacterium and hyphae. (Bar = 100 nm)



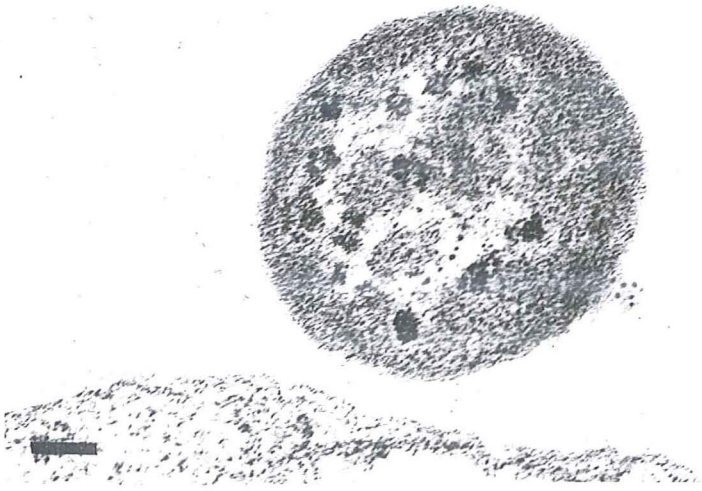


Fig. 3.28. Low power micrograph of *P. putida* PMS118R adhering to *A. bisporus* hypha (30 min). (Bar = 500 nm)

Fig. 3.29. High power micrograph of a single bacterial cell from Fig. 3.28. Clearly evident are the fibrillar rods which enable contact between the bacterium and the external polysaccharide layer of the hypha. (Bar = 100 nm)

Fig. 3.30. *P. putida* PMS118R attached to *A. bisporus* hypha (30 min). Note the uneven surface of the bacterium. (Bar = 100 nm)

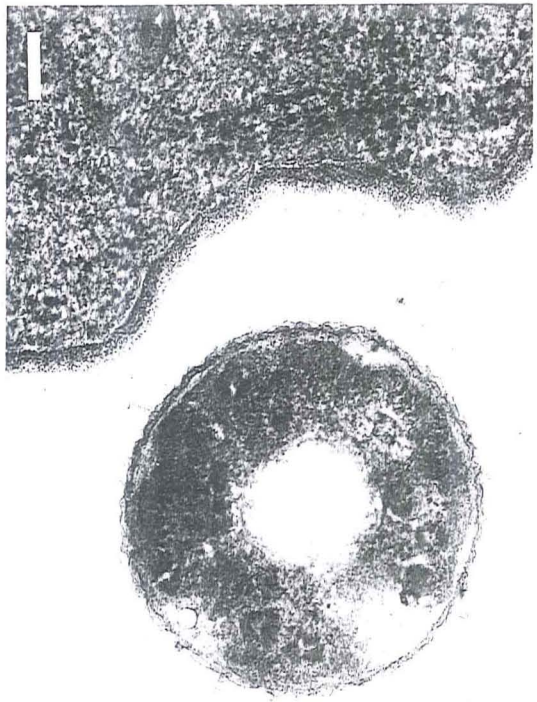
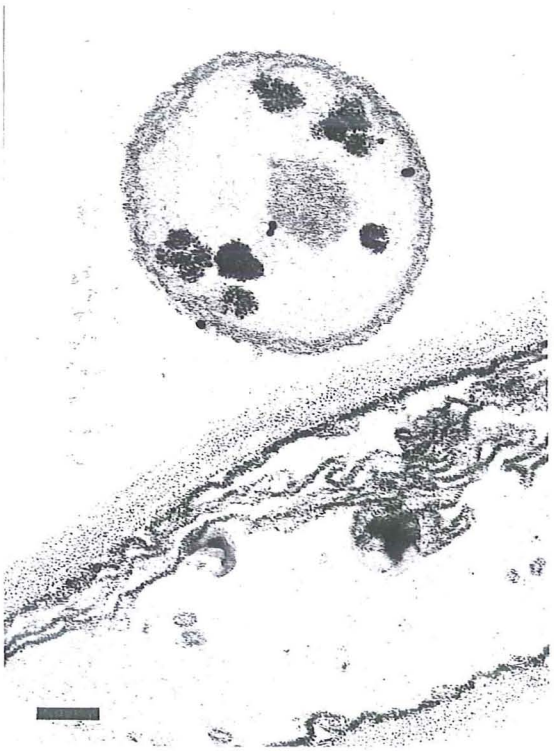


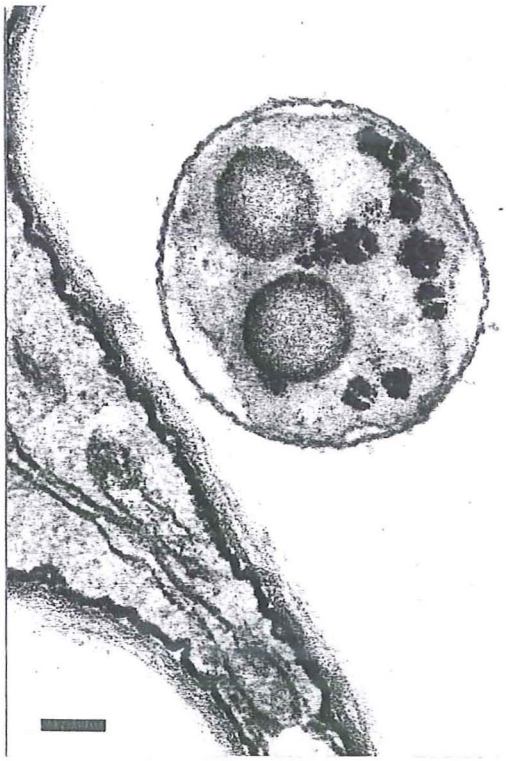
Fig. 3.31a. *P. putida* PMS118R attached to *A. bisporus* hypha (30 min) (TCH stained). Fibrillar rods are continuous with the outer surface of the bacterium and stain positive for polysaccharide material. (Bar = 100 nm)

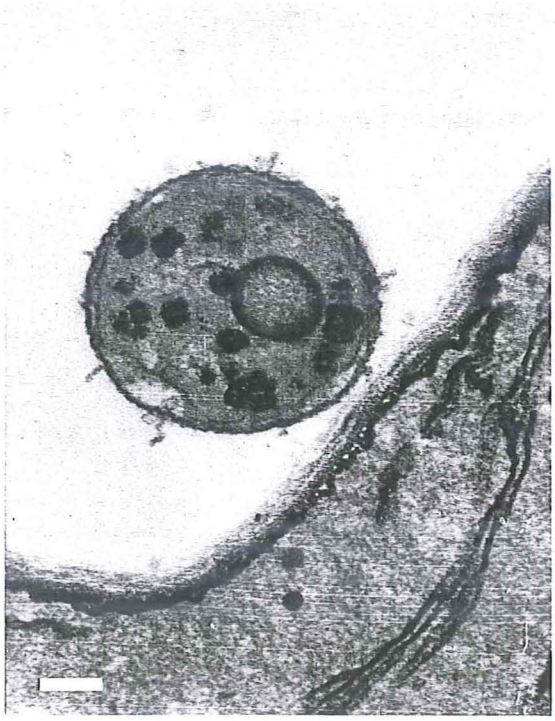
Fig. 3.31b. *P. putida* PMS118R attached to *A. bisporus* hypha (3 h) (TCH stained). Positive TCH-stained fibrillar rods connect the bacterium with the hyphae. (Bar = 100 nm)

Fig. 3.32a. *P. putida* PMS118R attached to *A. bisporus* hypha (3 h) (TCH stained). (Bar = 100 nm)

Fig. 3.32b. *P. putida* PMS118R attached to *A. bisporus* hypha (3 h). (Bar = 100 nm)







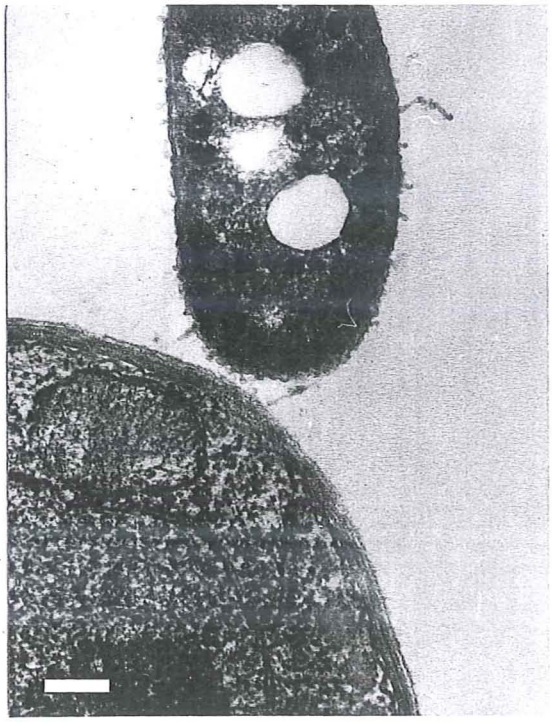
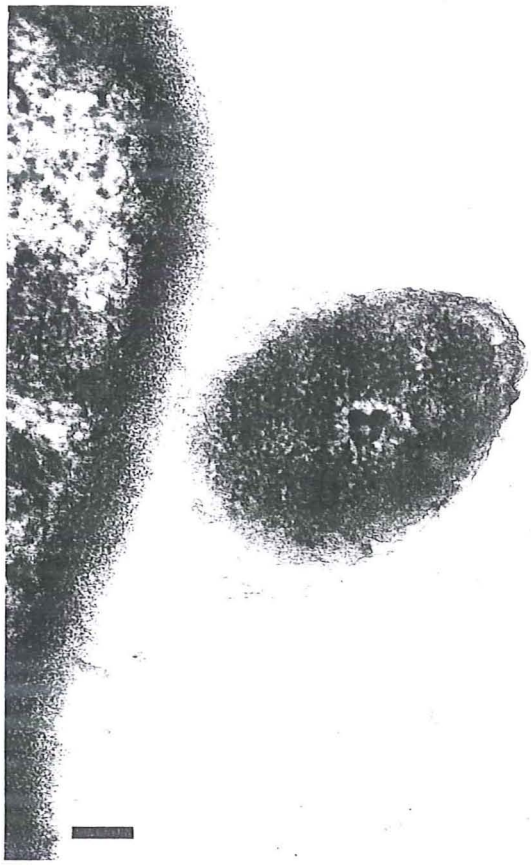


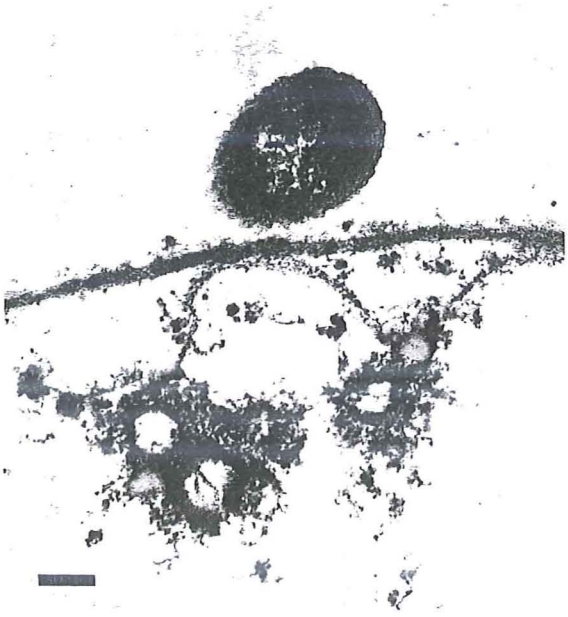
Fig. 3.33. *P. putida* PMS118R attached to *A. bisporus* hypha after 24 h. Long fibrillar rods protrude from the bacterial surface and anchoring material can be seen joining the bacterium with the hyphal surface. (Bar = 100 nm)

Fig. 3.34. *P. tolaasii* PMS117S attached to *A. bisporus* hypha (24 h) The plasma membrane has pulled away from the cell wall. (Bar = 100 nm)

Fig. 3.35a. *P. tolaasii* PMS117S attached to *A. bisporus* hypha (24 h). The plasma membrane has pulled away from the cell wall in places and the hyphal contents are largely missing. (Bar = 200 nm)

Fig. 3.35b. A section through a dolipore septum. Structure shows the parenthosome cap (p) and electron dense pore plugs (e). (Bar = 200 nm)






p
e

3.4 DISCUSSION

3.4.1. CHEMOTAXIS

Two pseudomonads frequently found in association with *A. bisporus*, *P. putida* and *P. tolaasii*, were shown to be attracted to mycelial exudates of this fungus, *in vitro*. Migration of these bacteria in casing soil toward *A. bisporus* mycelium growing on compost was also demonstrated. These results provide good evidence for the attraction of *P. putida* PMS118 and *P. tolaasii* PMS117 toward *A. bisporus* mycelium in the casing layer.

Cresswell & Hayes (1979) demonstrated that pseudomonads were the dominant group of bacteria within the casing layer and that the numbers of these bacteria were dependent upon the growth and fruiting cycle of *A. bisporus*. In a nutrient poor environment the production of metabolites by *A. bisporus* would create nutrient gradients to which chemotactic bacteria would respond. The ability to move quickly toward these nutrients would probably provide pseudomonads with a survival advantage over non-motile or non-chemotactic bacteria (Lauffenburger *et al*. 1981, Ames & Bergman 1981) and may partly explain the dominance of this group of bacteria within the casing layer.

The positive response exhibited by *P. putida* PMS118 toward mycelial exudates provides further evidence for its involvement in the process of basidiome initiation. The migration toward mycelial exudates may aid in the subsequent colonization of the hyphae by *P. putida* which appears necessary before the bacterium can provide *A. bisporus* with the stimulus necessary to trigger basidiome initiation. The similarity in the responses of the smooth and rough colonial forms to the exudates possibly reflects their equal ability to promote basidiome initiation (see Chapter 2.0.).

The information obtained from heat treating the exudate indicates that thermostable compounds are a source of attractants for both colonial forms, of both species. The decrease in the attraction of *P. tolaasii* PMS117S to the heated exudate, suggests that some heat labile compounds also attract this bacterium. The failure of the rough and smooth colonial forms of *P. putida* PMS118 and the rough colonial form of *P. tolaasii* PMS117 to respond to the dialyzed

exudate indicates that the thermostable attractants for these isolates are of low (< 10,000 MW) molecular weight.

The increased attraction of the rough colonial forms of *P. putida* PMS118 and *P. tolaasii* PMS117 toward the heat treated exudate, suggests that the untreated exudate may contain some heat labile compounds which serve as repellents for these forms. The nature of these compounds is not known, but it is possible that they may be bacteriolytic enzymes (Grant *et al.* 1984), or other heat labile antimicrobial compounds produced by *A. bisporus* mycelium (Claydon 1985). Lipopolysaccharides of rough colonial forms have been shown to differ both quantitatively and qualitatively, from those of the smooth colonial forms (Jarrell & Kropinski 1977, Gerwe *et al.* 1987). The increased sensitivity of the rough colonial forms may be a result of the altered lipopolysaccharides which may not provide the protection to antimicrobial compounds afforded by the lipopolysaccharide layer of the smooth colonial forms.

Smooth, pathogenic, *P. tolaasii* PMS117 was attracted to both the heat-treated and dialyzed exudates, which indicates that both large (> 10,000 MW) and thermostable compounds serve as attractants. This contrasts with the rough colonial form, which was attracted only toward small molecular weight, thermostable compounds. The differences in the nature of the attractants for the two *P. tolaasii* colonial forms is interesting and may be significant in terms of the poor capacity of the rough, non-pathogenic form to adhere to *A. bisporus* hyphal surfaces (section 3.4.2.).

The attraction of bacteria to macromolecules is unusual (Currier & Strobel 1977), but suggests that *P. tolaasii* PMS117S may possess a specific means of recognizing its host. More work is required to determine the nature of this attractant(s), but if specific for *P. tolaasii*, then the development of a mushroom strain unable to produce this compound, or the controlled release into the casing layer of the attractant, would provide novel approaches for the control of bacterial blotch disease.

3.4.2. ADHERENCE

Quantitative studies

The results obtained from this section of work demonstrate the ability of *P. putida* PMS118 and *P. tolaasii* PMS117 to adhere rapidly and firmly (that is, cells were not removed by washing), to *A. bisporus* mycelium.

The percentage attachment of the smooth colonial form of each species to *A. bisporus* mycelium, after growth on KB, was similar to that reported by Preece & Wong (1982) for attachment of these bacteria to discs of cap mycelium, after growth on nutrient agar. Preece & Wong (1982) reported a percentage attachment value of 31 % for *P. putida* and 74 % for *P. tolaasii* and concluded that the attachment process, in quantitative terms, differed markedly between pathogens and non-pathogens. The results from this study show that after growth on media more representative of the nutrient status of the casing layer than nutrient agar, or KB, the difference between the percentage attachment of *P. putida* and *P. tolaasii* was not marked. This highlights the importance of conducting attachment assays under conditions which approximate the situation *in vivo*. It also indicates that care should be taken when interpreting the results of *in vitro* attachment studies.

The percentage attachment of bacteria to mycelium after growth on media of different nutrient composition and status, provides some information on the attachment process. The composition and quantity of polysaccharide surface polymers produced by Gram-negative bacteria is influenced by the proportion and form of carbon and nitrogen in the growth substrate (see Sutherland 1985). Exopolysaccharide synthesis is greatest under conditions of nitrogen limitation, therefore, if these polymers are predominantly responsible for adhesion, then attachment would be expected to be greatest after growth on N-limited media. The percentage attachment of both forms of *P. putida* PMS118 after growth on N-limited M63 did not differ from the percentage attachment after growth on KB or M63 (Fig. 3.1) which indicates that production of large amounts of polysaccharide material does not enhance the adhesive properties of these cells.

Brown *et al.* (1977), Pringle *et al.* (1983) and Rosenberg *et al.* (1983) have also shown, in contrast to previous belief, that production of exopolysaccharide material does not always promote attachment. Brown *et al.* (1977) noted more effective attachment of bacterial cells under C-limited conditions and Rosenberg *et al.* (1983) showed that capsulated *Acinetobacter calcoaceticus* did not adhere to hydrocarbon, whereas enzymatically decapsulated isolates were strongly hydrophobic and adhered in large numbers. Pringle *et al.* (1983) suggested that hydrophilic polysaccharide material was concerned with the development of bacterial films, rather than the initial attachment process.

On C-limited media, the percentage attachment of the rough colonial form of *P. putida* PMS118 was similar to the percentage attachment of this form after growth on KB, M63 and N-limited M63, but the percentage attachment of the smooth colonial form increased. This may be due to the production of polysaccharide material which has better adhesive properties, or may be due to exposure of hydrophobic regions on the bacterial surface, possibly membrane bound proteins, which were previously masked by the presence of excess polysaccharide.

Decreasing the concentration of carbon and nitrogen in M63 to 0.1 % resulted in increased attachment of both forms of *P. putida* PMS118. This result is probably related to the small size of the starved cells, which are known to have a greater tendency for firm adhesion (Dawson *et al.* 1981 and Kjelleberg *et al.* 1983).

The different attachment patterns of the various colonial forms of *P. putida* PMS118 have important ecological implications for the survival of this organism. This is particularly apparent when the total population is considered. No single cell is able to express the multiplicity of forms which are found in a population, but the progeny of a single cell includes forms which possess different adhesive properties. In effect, a population which possess cell-types able to adhere under a range of different conditions is pre-adapted to changes in the environment (Silverman *et al.* 1984). The production of a range of colonial forms will enable *P. putida* to adhere to *A. bisporus* mycelium over a greater range of environmental conditions.

The attachment of *P. putida* PMS118S to *A. bisporus* mycelium was significantly increased when $CaCl_2$ was present. Similar cations effects have been reported for adherence of *P. tolaasii*

to barley roots (Nissen 1973), adherence of *P. aeruginosa* to steel (Stanley 1983), adherence of a marine pseudomonad to glass (Marshall *et al.* 1971) and adherence of *P. fluorescens* to radish roots (James *et al.* 1985). The effect of the calcium cation is thought to indicate involvement of electrostatic forces in the attachment process (Marshall *et al.* 1971). Cations are thought to mask the repulsion caused by negatively charged acidic polysaccharides on both the surface of the bacteria and fungus and allow the organisms to approach, and in some cases adhere to one another (James *et al.* 1985). Cation-stimulated binding appears to play an important role in rapid, firm adhesion of the smooth colonial form of *P. putida* PMS118 to *A. bisporus* mycelium.

The apparent involvement of calcium in the adherence of *P. putida* PMS118 to mycelium has important implications for the adhesion of this bacterium to mycelium in the casing layer. In this environment large amounts of calcium are present, both dissolved (Rainey 1985) and as calcium oxalate crystals on hyphal surfaces (Masaphy *et al.* 1987). It is likely that Ca^{2+} aids attachment of bacteria which utilize electrostatic forces to facilitate adherence.

The apparent involvement of electrostatic forces also indicates that the smooth colonial form of *P. putida* PMS118 may possess more than one mechanism of adhesion. A high percentage attachment was found after growth on media where polysaccharide production was limited and after growth on media where the production polysaccharide was enhanced, provided the calcium cation was present. More than one mechanism of attachment has also been indicated for a marine vibrio (Paul & Jeffery 1985a). The ability to adhere to surfaces under a range of different conditions may confer a survival advantage on this organism.

Attachment of the smooth colonial form of *P. putida* PMS118 to mycelium after growth on CSLM could be due to a combination of factors (as discussed above), but the high percentage attachment of the rough colonial form is not readily explained. Nevertheless, the ability of this form to attach to mycelium under conditions of nutrient limitation, such as those which exist within the casing layer, was demonstrated.

Complete inhibition of attachment of both colonial forms of *P. putida* PMS118 by the surfactant, Triton X-100, a disruptor of hydrophobic interactions, suggests that these interactions are important in the adhesion process. Paul & Jeffrey (1985b) also found that dilute

Triton X-100 completely inhibited attachment of estuarine and marine bacteria to hydrophobic surfaces, but reported that it had little effect on the adsorption of bacteria to glass, a hydrophilic substrata. This result appears to conflict with the measures of hydrophobicity reported in section 3.3.2., which indicated that the bacterial isolates are only moderately hydrophobic. However, the nature of the surface to which bacteria adhere has a marked effect on the adhesion process (Fletcher & Loeb 1979) and this result possibly reflects the hydrophobic nature of the mycelial surface. Hydrophobic interactions tend to result from the presence of proteinaceous adhesins. It would be interesting to see if inhibitors of protein synthesis and/or proteolytic enzymes cause a decrease in adherence of *P. putida* to hyphae.

The smooth colonial form of *P. tolaasii* PMS117 attached to the mycelium in large numbers, regardless of the medium on which it was cultured. The greatest percentage attachment was observed after growth on N-limited M63 which suggests that polysaccharide material plays an important role in adhesion of this organism to *A. bisporus* mycelium. The presence of the calcium cation caused a slight decrease in the percentage attachment of *P. tolaasii* PMS117S which suggests that electrostatic forces may not be involved in the attachment process. The low percentage attachment of the rough colonial form of *P. tolaasii* PMS117 under all conditions is unusual, but is consistent with its known inability to produce a toxin and cause disease of the mushroom (Cutri *et al.* 1984).

While the question of specificity has not been directly examined in this study, the discovery that *P. tolaasii* PMS117R adheres poorly to mycelium while the smooth colonial form of *P. tolaasii* PMS117, and both colonial forms of *P. putida* PMS118 are able to attach in large numbers, suggests that there may be some specificity in the interaction between certain bacteria and the mycelium. This requires further study and the attachment assay could be used for this purpose. Quantitative time course experiments would undoubtedly yield much useful information, as would quantitative assays conducted in the presence of known receptor blockers, such as simple sugar molecules. The attachment assay could also be used to examine the ability of *P. putida* to attach to the mycelium in the presence of other bacterial species.

Microscopic studies

The results from the SEM study confirmed the findings of the quantitative attachment work described above. The large differences in the attachment of *P. aeruginosa* OT11 and *P. syringae* PDDCC7607 to *A. bisporus* mycelium also suggests that some degree of specificity may exist in the association of certain bacteria with *A. bisporus* mycelium.

Fibrillar rods associated with the attached cells were also reported by Preece & Wong (1982) who found them joining *P. tolaasii* cells to each other, and to *A. bisporus* hyphae. Preece and Wong (1982) also concluded that they did not resemble flagella, or fimbrae and produced evidence which suggested that they were not artifacts of preparation and fixation. Similar structures have been found anchoring *Agrobacterium* to plant cells (Matthysse 1983, Graves *et al*. 1988) and lactobacilli to chicken crop epithelium (Fuller & Brooker 1980). Brooker & Fuller (1975) were able to show that the fibrillar material had the staining characteristics typical of carbohydrate material. Fibrillar like material has also been observed linking *Rhizobium* cells to *Phytophthora megasperma* hyphae (Tu 1979) and to *P. cinnamomi* (Malajczuk *et al*. 1984). Malajczuk *et al*. (1984) referred to this material as 'slime strands'.

Uneven distribution of *P. tolaasii* cells on *A. bisporus* hyphae was also reported by Preece & Wong (1982) and uneven distribution of *Agrobacterium* cells on cut plant surfaces was noted by Graves *et al*. (1988). It is possible that the distribution of cells reflects metabolically active hyphae, and/or hyphae with particular surface characteristics, but this requires further investigation.

When *P. putida* PMS118 and *P. tolaasii* PMS117 were incubated with *A. bisporus* under nutrient replete conditions, the number of rough *P. putida* PMS118 cells attached after 24 h appeared to decline, while the number of smooth cells attached to the mycelium, of both species, increased. The nutrient rich conditions promoted the production polysaccharide slime material which appeared to facilitate permanent adherence of the smooth colonial forms, while leading to the detachment of the rough colonial forms. The detachment of bacteria following the production of polysaccharide material has been reported by Rosenberg *et al*. (1983) and they suggested that

this behaviour enabled bacteria to leave a colonized surface in search of fresh, unexploited interfaces.

When attachment was allowed to proceed longer than 24 h under nutrient poor conditions, the opposite response was observed. The number of bacteria of the smooth colonial forms attached decreased markedly while the number of the rough colonial form (of *P. putida* PMS118 only) appeared to increase. This may indicate a requirement of the smooth colonial forms for nutrients to enable continual production of polysaccharide material in order to remain permanently attached. It may also indicate the inability of the smooth colonial forms to withstand nutrient poor conditions to the same extent as the rough colonial forms (see Chapter 2.0.). The production of micro-colonies by *P. putida* PMS118S presumably resulted from the growth and division of a single attached cell. It would have been interesting to have cultured these cells on agar media to determine the nature of the colonial form that these cells possessed. The lack of fibrillar material associated with the rough colonial forms suggests that this material is not important during this stage of the attachment process, while the perpendicular orientation of the rough cells suggests the existence of a specialized attachment region on the bacterium. Polar attachment of *Rhizobium* cells to clover root hairs is observed during the last stage of phase I attachment, after the bacterial capsule has been modified by enzymes released from the clover root (Dazzo *et al.* 1982).

The discovery of *P. putida* PMS118S cells within sections of hyphae was interesting, but it is not known whether the cells entered through a wall rupture, or whether they actively penetrated the cell. Intra-cellular bacteria have been reported in *A. bisporus* (see Oxley 1986), but it is most likely that *P. putida* PMS118S entered the hyphae via a break in the wall.

The production of capsules by *P. putida* PMS118 in response to *A. bisporus* mycelium was unusual, as fluorescent pseudomonads do not typically produce these structures (Palleroni 1984). Of particular interest was the way in which the capsulated bacteria engulfed the hyphae. The significance of this is not known, but the perpendicular orientation of the cells, again suggests a specific attachment point on the bacterium and parallels can be drawn between this

and the initial phase of the *Rhizobium*-legume interaction. It would be of value to investigate this phenomenon further.

The TEM study revealed useful information on the mechanism of attachment of the bacteria to the mycelial surfaces. All of the isolates, with the exception of *P. tolaasii* PMS117R (which was not seen by TEM), possessed rough, uneven, preformed extracellular polymers on their external surfaces. These were particularly obvious in cells of *P. putida* PMS118R, where uneven fibrillar rods were frequently visible. The fibrillar appendages and uneven surface polymer layers gave a positive reaction to TCH staining confirming the presence of polysaccharide material. Large amounts of polysaccharide material also surrounded the hyphae and probably plays an important part in the adhesion process. The uneven surface appendages, in conjunction with available cations, are probably largely responsible for the rapid firm adhesion of the pseudomonads to hyphal surfaces. These structures would enable bacteria to overcome the repulsion energy barrier and achieve rapid (within 30 min), firm attachment to the hyphal surface (Fletcher 1980).

The build-up of polysaccharide material between *P. putida* PMS118 and *P. tolaasii* PMS117 and hyphae appears to be responsible for anchoring the bacteria firmly to the surface (Marshall *et al.* 1971, Fletcher 1980). It would be interesting to know if this material arose solely from the bacterium, or whether the fungus also contributes polysaccharide material which helps cement the bacterium to its surface.

The results discussed above indicate the complexity of the adhesion process. *P. putida* PMS118 and *P. tolaasii* PMS117 appear to attach to the mycelium by a range of mechanisms, involving hydrophobic interactions and electrostatic forces. Attachment occurs rapidly and firmly, probably a result of the uneven polysaccharide polymers on the bacterial surfaces, but facilitated by the presence of the calcium cation. After the initial rapid adsorption stage, cells may, depending on their surface properties, either consolidate their position through the production of polysaccharide polymers, or free themselves from the hyphal surfaces, apparently also by polymer production.

The ability of *P. putida* PMS118 to attach to mycelial surfaces under a range of conditions is important if the bacterium is to stay in contact with the fungus. Remaining in contact with the fungal mycelium is most likely to the advantage of the bacterium as this would assure it of a supply of utilizable metabolites and a secure niche. By allowing (and perhaps encouraging) this association, the fungus ensures that the organism responsible for providing it with the reproductive stimulus is constantly present. In addition to stimulating reproductive growth, it is possible that *P. putida* may also serve a protective role by efficiently colonizing the hyphal surfaces and excluding pathogenic organisms, in a manner similar to the beneficial rhizosphere pseudomonads (see Kloepper *et al*. 1988). This requires further investigation, but if shown to be true, would help to explain why *A. bisporus* has come to rely on the presence of another organism in order to reproduce.

CHAPTER FOUR

THE INVOLVEMENT OF *PSEUDOMONAS PUTIDA* IN BASIDIOME INITIATION OF *AGARICUS BISPORUS*

4.1. INTRODUCTION

4.1.1. THE INVOLVEMENT OF *P. PUTIDA* IN BASIDIOME INITIATION OF *A. BISPORUS*

The involvement of *micro*-organisms in basidiome morphogenesis of *A. bisporus* was first reported by Eger (1961). Using a simple laboratory method for the production of basidiomata *in vitro*, the 'Halbschalentest', Eger found that fruit body initiation was inhibited when *A. bisporus* was grown axenically. Application of a suspension of casing layer derived micro-organisms to the sterile casing restored the basidiome stimulatory properties of the casing layer. Eger also reported that activated charcoal, when incorporated into a sterile casing layer, could replace the effects of micro-organisms. Since activated charcoal is a known absorbent of low molecular weight organic chemicals, Eger suggested that the bacteria controlled mushroom fruiting by removing 'self-inhibitory compounds', produced by the mushroom mycelium, to below a threshold level which enabled fruiting to occur. The ability of activated charcoal to promote basidiome initiation in axenic systems was confirmed by Couvy (1974), Long & Jacobs (1974), Wood & Goodenough (1977) and Peerally (1979). Long & Jacobs (1974) also demonstrated that a decrease in the concentration of carbon dioxide to below 0.1 % (which is necessary for reproductive growth (Long & Jacobs 1969)) did not stimulate fruiting unless the casing soil contained either a viable microflora, or activated charcoal.

The micro-organisms responsible for initiating basidiome development were isolated by Hayes *et al.* (1969) who added known volatile metabolites of *A. bisporus*, including ethanol, ethyl acetate and acetone, to enrichment cultures. They found that after 5 d incubation, bacteria closely related to *P. putida* predominated within the culture flasks. These isolates were able to promote basidiome initiation when applied to sterile casing soil. The ability of *P. putida*, isolated from the mushroom casing layer, to promote mushroom fruiting was confirmed by Rainey & Cole (1987).

O'Donoghue (1962) observed basidiome initiation in what was thought to be a pure bag of spawn, but closer examination revealed the presence of actinomycetes which were presumed responsible. Curto & Flavelli (1972) reported that yeasts and micro-algae increased basidiome

production and mycelial density and Urayama (1967) described a metabolite isolated from *Bacillus psilocybe* which induced basidiome formation in a range of agarics. This isolate, however, has since become ineffective (as quoted by Hayes *et al.* (1969)). Isolates of *P. putida* able to promote initiation of *A. bisporus* in compost grown cultures were claimed by Hume & Hayes (1972) to stimulate primordium formation when both fungus and bacterium were cultured together on 2 % malt extract agar (MEA). Wood (1976) examined the same bacterial isolates and used an identical laboratory system, but was unable to achieve these results. He did however observe axenic fruiting of commercial *A. bisporus* strains on 2 % MEA and found that the ability of these strains to form primordia differed significantly between strains and within each strain, and was dependent on the agar source.

Park & Agnihotri (1969a, 1969b) claimed that in addition to bacteria, culture filtrates and a range of chemicals, including biotin and oxalic acid, could stimulate basidiome development, but Eger (1972) and Wood (1976) were unable to confirm these results.

Hayes (1972) reported that iron containing compounds and chelating agents, when added to axenically grown plate cultures of *A. bisporus*, were also able to promote fruiting and suggested that bacteria, in particular *P. putida*, controlled fruiting by releasing iron from organic chelating agents in the casing layer. Hayes made no claim to the identity of these compounds, but as suggested by Wood (1976), it is most likely that they would be analogous to the low molecular weight, ferric iron specific ligands, known as siderophores (see section 2.2.3.). Wood (1976) was unable to stimulate fruiting by applying a large range of iron containing compounds, free chelating agents and iron containing chelating agents, to *A. bisporus* cultures.

Hayes re-examined the role of bacteria with respect to iron and basidiome initiation and in 1981 reported that growth of *A. bisporus* vegetative mycelium through the casing layer caused the amount of soluble iron within this environment to increase. He suggested that the water soluble iron (produced by the mushroom mycelium and possibly inhibitory to its own growth (Hayes 1972)) was 'fixed' by bacteria into an insoluble form which maintained levels of water soluble iron at concentrations favourable for the growth and fruiting of the mushroom.

Wood (1982) attempted to isolate the putative self-inhibitors thought to be produced by the mushroom mycelium by growing cultures of *A. bisporus* axenically in the presence of activated charcoal. The chemicals binding to the charcoal were identified by coupled gas chromatography-mass spectroscopy and pure samples of these compounds were bioassayed by placing them adjacent to colonies of plate grown cultures of *A. bisporus* or *A. bitorquis* (Quel) Sacc. Two compounds were found to effect primordia formation, 1-octen-3-ol inhibited fruiting, while benzyl alcohol stimulated fruit body initials. Wood was unable to relate the effects of these compounds on basidiome initiation to the putative inhibitors, as the concentrations at which these compounds are physiologically active is not known, nor are the concentrations of these compounds within the casing layer. Furthermore, 1-octen-3-ol is the major flavour component of the mushroom (Pyysalo & Suikko 1976) and it therefore seemed unlikely that it would have a role in fruit body initiation (D. A. Wood, personal communication).

Little direct evidence has been presented for theories explaining the mechanism by which bacteria, particularly *P. putida*, trigger basidiome initiation. The stimulus provided by *P. putida* may be either positive, by production of a fruit inducing substance (Urayama 1967, Hayes 1972), or negative, through removal of an inhibitory compound produced by the mycelium (Eger 1961), but the ability of activated charcoal to replace the effects of micro-organisms, suggests that the stimulus is negative (Wood 1976, 1982). The 'self-inhibitor' theory, first put forward by Eger (1961), also provides a means of explaining the occasional development of primordia under axenic conditions, where air or water could function in removing inhibitors (Rainey and Cole 1987).

4.1.2. THE EFFECT OF BACTERIA ON MYCELIAL GROWTH

Confusion has arisen concerning the effect of bacteria on mycelial growth, prior to the appearance of basidiomata. This has occurred because workers have failed to differentiate between inoculation of *A. bisporus* with mixed bacterial cultures and single isolates (Rainey & Cole 1987). Eger (1961, 1962) and Peerally (1979) inoculated pure fungal cultures growing on compost/casing soil with mixed bacterial populations and noted that mycelial growth was inhibited and that fruiting was associated with this check in growth. They also reported that

coarse mycelial strand development was promoted. When single isolates of *P. putida* were applied to *A. bisporus* grown under similar conditions, Urayama (1967), Eger (1972) and Rainey & Cole (1987), found that the rate of linear extension of vegetative mycelium was stimulated by *P. putida*, prior to the appearance of basidiome initials and that coarse mycelial strand formation was not promoted. Rainey & Cole (1987) suggested that coarse strand development and inhibition of mycelial growth was caused by bacteria within the casing layer other than *P. putida*.

The increase in the rate of hyphal extension stimulated by *P. putida* is presumably accompanied by an alteration in the frequency of branching. Alterations in the rate and manner in which primary hyphae extend and branch determine the morphology of fungal colonies (Bull & Trinci 1977) and result in the formation of specialized structures, including basidiomata, sclerotia, mycelial cords and rhizomorphs (Corner 1948, Hock *et al.* 1978, Willets 1978, Watkinson 1979). Stimulation of the rate of hyphal extension by *P. putida* may represent an early stage in the morphogensis of basidiome initials. If this is so, then the ability to delimit this phase from the later stage of primordium initiation and to examine it in isolation, would considerably aid the study of fruit body initiation of *A. bisporus*.

4.1.3. *IN VITRO* GROWTH AND FRUITING OF *A. BISPORUS*

Studies investigating the influence of bacteria, in particular *P. putida*, on the process of basidiome initiation are severely limited by weak vegetative growth and poor basidiome initiation of the fungus in Petri dish culture.

A range of artificial substrates are commonly used for the growth of *A. bisporus*, including malt extract agar (MEA; 2-4 % malt extract) (Hume & Hayes 1972, Wood 1976, San Antonio & Thomas 1972, Mathew 1961, Rainey & Cole 1987), potato dextrose agar (PDA) (Peerally 1979), complete yeast medium (CYM) (Elliott & Wood 1978), and commercially prepared malt extract (peptone) agar (MPA) (Masaphy *et al.* 1987), but the growth of the mycelium on these media is slow, and sectoring and strain degeneration are commonly seen (see plates in Wood (1976)). Furthermore, phenomena observed on these media do not always reflect the situation *in vivo* (Rainey & Cole 1987). Media utilizing powdered compost, or compost extracts are sometimes

used by spawn manufacturers and are reported to promote even growth (Fritsche 1978). These substrates, however, are rarely used for laboratory studies.

Methods for the production of primordia *in vitro* have been described (Eger 1961, Hume & Hayes 1972, Long & Jacobs 1974, Peerally 1979, 1981, Rainey & Cole 1987), but are either elaborate and time consuming to set up, or unreliable (Wood 1976, Rainey & Cole 1986). Strains of *A. bisporus*, such as developmental variants B430 and B431 (Elliott & Wood 1978), and of *A. bitorquis* (a species taxonomically and morphologically close to *A. bisporus*) which readily produce primordia in Petri dish culture (Raper 1976), have been used to study some of the cytological and biochemical events of initiation (Wood 1979). However, since these strains initiate fruit bodies in axenic culture, on agar media, they are of limited value for studies on the nature of the microbial stimulus affecting basidiome initiations associated with most commercial strains.

A laboratory medium which promotes rapid, vigorous growth of the mycelium and a mushroom strain able to consistently produce primordia when cultured in association with basidiome stimulatory bacteria are required before investigations into the biology of the interaction between *P. putida* and *A. bisporus* can proceed.

4.2. MATERIALS AND METHODS

4.2.1. STRAINS

Fungal strains

A commercial *A. bisporus* hybrid strain, 'Horst U3' (Somycel) was used throughout. W19 is a wild tropical strain of *Agaricus* with commercial potential (Smith & Love 1987) and was obtained from the culture collection of the Institute of Horticultural Research (I.H.R.), Littlehampton, West Sussex, U.K. W19 is reported to be typical of *A. bitorquis*, but has an optimal temperature for growth and fruiting of 30 °C (5 - 7 °C higher than commercial *A. bitorquis* strains) and exhibits a cropping cycle similar to *A. bisporus* (Smith & Love, in press).

A. bisporus (strain 649), *A. campestris* L. ex Fr., *A. bitorquis* (strain W2), *Auricularia auricula* (Hook) Underw, *Coprinus bilanatus*, *Flammulina velutipes* (Curt. ex Fr.) Karst., *Lentinus edodes*, *Pleurotus ostreatus* (Jacquin ex Fr.) Kummer , *P. flabelatus*, *P. cystidiodes*, *P. eaus* and *Volvariella volvacea* (Bull. ex Fr) Singer were also obtained from I.H.R.

Maintenance of fungal strains

Fungal strains were maintained on washed mushroom compost at 4 °C. This substrate was prepared by pouring 1 l of hot (100 °C) distilled water over 50 g of fresh commercial compost contained within a muslin bag. The drained compost was placed in glass flasks and sterilized by autoclaving (120 °C, 1 h, 1.2 atm) on three successive days.

Bacterial strains.

Pseudomonas strains were isolated from the casing layer of commercial mushroom farms located in Christchurch, New Zealand, and are characterized in Chapter 2.0. Isolates were maintained in nutrient broth, 20 % glycerol, at -80 °C and when required for experimentation were grown overnight in an orbital incubator at 28 °C in 10 ml of KB broth. Isolates were washed twice in sterile PBS and suspended in sterile distilled water (10^8 cells ml^{-1}) before inoculating onto

mushroom strains. *Arthrobacter flavescens* (JG-9) (Lochhead *et al*. 1952) was obtained from I.H.R.

4.2.2. DEVELOPMENT OF A LABORATORY SUBSTRATE SUITABLE FOR CULTURE OF *A. BISPORUS*

Fungi are often grown on artificial media which bear little resemblance to their natural substrate (Tribe 1987). Media commonly used for *in vitro* culture of *A. bisporus* are devoid of insoluble material and are rich in simple sugars, few of which are encountered by the mushroom on its commercial substrate. *A. bisporus* is usually grown on composted wheat straw/horse manure, a material which consists of a mixture of complex, mainly insoluble, plant and microbial residues. The insoluble fraction, which includes lignin, cellulose, hemicellulose, protein and microbial biomass, is preferentially used by the mushroom mycelium for growth (Fermor & Wood 1979).

The microbial biomass represents approximately 2 % of the compost dry weight (Sparling *et al*. 1982) and its importance as a source of nutrients for *A. bisporus* has recently been demonstrated (Fermor & Wood 1981, 1982, Grant *et al*. 1984, Fermor & Grant 1985). During composting, a dark brown, amorphous matrix of polysaccharide, microbial cells and debris, accumulates on the straw surfaces (Eddy & Jacobs 1976, Atkey & Wood 1983). *A. bisporus* produces a range of extracellular enzymes, including bacterial cell wall degrading muramidases, which enable it to utilize this 'microbial matrix' as a concentrated source of nitrogen, minerals and carbon (Fermor & Grant 1985).

The following medium (compost malt medium (CMM)) was developed after consideration of the commercial substrate and growth requirements of the mushroom. The contribution of the insoluble fractions from the compost, especially the microbial biomass, to the nutrition of the fungus were considered important. The formulation described below was adopted after conducting preliminary trials which examined the effect of a range of CMM components on the growth of *A. bisporus*.

Compost malt medium (CMM)

A sample (5 - 10 kg) of fresh, evenly textured, commercially prepared, mushroom compost was oven dried (75 ^{o}C for 2 days, or until no further weight loss was detected) and stored at room

temperature. A 50 g sub-sample (70 g when preparing the medium for *A. bitorquis* W19) of the dried compost was added to 1 l of distilled water and the mixture left to infuse for 1 h. The temperature was then raised to 100 °C for 5 min and the mixture stirred vigorously to aid removal of the 'microbial matrix' from the straw surfaces. After a further 2 h infusion period the cooled mixture was filtered through four layers of muslin and approximately 800 ml of dark brown infusate was recovered. To this liquid 0.75 % malt extract (Oxoid L39) and 1.5 % Bacto agar (Difco B140) were added, and the pH was adjusted to pH 7.4 with 1 N NaOH. The medium was autoclaved (121 °C, 15 min, 1.2 atm) and mixed well to ensure even distribution of the particulate matter, before pouring into Petri dishes.

The suitability of CMM as an artificial substrate for the growth of *A. bisporus* was examined by comparing the radial extension rate of *A. bisporus* mycelium on CMM and on a range of frequently used laboratory media. The vigour, uniformity of growth and sectoring tendencies of mycelium were also assessed. The following media were used:- 2 % MEA-Bacto (2 % Bacto malt extract; Difco B186, 1.5 % Bacto agar; Difco B140), 2 % MEA-Maltexo (2 % 'Maltexo' malt extract; Cerebos Gregg's Ltd., New Zealand, 1.5 % Bacto agar; Difco B140), PDA (Oxoid CM139), MPA (Oxoid CM59 - contains 0.5 % peptone), the basal medium of Eggins & Pugh (1962) supplemented with 1 % glucose and solidified with 1.5 % Bacto agar (Difco B140) and CMM as described above. CMM from which the malt extract was omitted (compost extract agar (CEA)) was also used in the trial. The pH of all media was adjusted to pH 7.4 with 1 N NaOH and sterilized by autoclaving. Disposable Petri dishes, 90 mm diam, were filled with 25 ml of medium (five replicates per treatment) and centrally inoculated with a 5 mm agar plug removed from the margin of a 10 d culture of *A. bisporus* growing on basal medium. Plates were incubated at 25 °C and colony diam (the average of 2 perpendicular measurements) measured at 48 h intervals.

4.2.3. *IN VITRO* FRUITING OF *A. BISPORUS*

A model system for examining the effect of bacteria on basidiome initiation

A range of *A. bisporus* and *A. bitorquis* strains retrieved from the culture collection at I.H.R. were examined for their ability to initiate basidiome development in Petri dish culture, in association with basidiome stimulatory isolates of *P. putida*. A strain of *A. bitorquis*, designated W19 (see section 4.2.1.), was found which possessed this ability.

Basidiome initiation of W19 in Petri dish culture.

Petri dishes containing 25 ml of CMM were inoculated with a 5 mm plug of W19 removed from the growing margin of a 10 d culture. A high carbon dioxide concentration, favourable for vegetative mycelial growth (Long & Jacobs 1969), was maintained within each Petri dish by sealing the lid with 'Parafilm M' (American Can Company Ltd., CT., USA). After incubating for 1 wk at 30 °C, bacterial isolates were inoculated onto the surface of the CMM; three, or four streaks per plate (see Figs 4.6 and 4.7). The Petri dishes were left unsealed and re-incubated at 30 °C and the relative humidity within the incubator was maintained between 70 and 80 %. After the mycelium had extended 5 mm over the bacterial colonies the temperature within the incubator was lowered to 20 °C for 24 h. This was not necessary for initiation of primordia, but ensured a more uniform fruiting response.

In vitro **systems for the production of basidiomata by** *A. bisporus*

The production of basidiomata by *A. bisporus in vitro* was achieved using two methods:- The Halbschalentest (Eger 1961) and a modification of the Halbschalentest, which approximates the commercial mushroom growing procedure.

Modified Halbschalentest.

Commercial mushroom compost was dried and milled in a blender to give a maximum straw length of less than 10 mm. The moisture content was adjusted to 75 % (wt/wt) and the compost sterilized by autoclaving (120 °C, 1 h, 1.2 atm) on 3 successive days. When cool, 75 g of compost was placed in a sterile 600 ml glass jar and inoculated with 1 g of *A. bisporus* rye-grain

spawn. A 90 mm diam glass petri dish lid was placed over the mouth of the jar and the container incubated at 25 °C until the compost was colonized (5 d). Sterile casing material (peat mixed with lime, 2:1 by volume and autoclaved for (120 °C, 1 h, 1.2 atm) on three successive days) was then applied to the surface of the colonized compost, to a depth of approximately 1.5 cm, and the jar re-incubated at 25 °C. Once the mycelium had begun to colonize the casing layer (3 - 4 d), 3 ml of bacterial suspension was applied to the surface of the peat. After a further 2 d incubation at 25 °C the carbon dioxide concentration within the vessel was lowered to favour reproductive growth (Long & Jacobs 1969). This was achieved by raising the lid 2 mm with small rubber lugs and suspending a 5 ml bottle containing 3 ml of 1 M KOH in the jar (Fig. 4.1). The container was re-incubated at 20 °C and the casing layer kept moist by applying sterile distilled water when necessary. The sterility of the casing material from control jars was checked at the end of experiments.

Halbschalentest.

The method of Eger (1961) was used. Dried compost was treated as described above and sterilized in 500 ml flasks. The compost was inoculated with approximately 1 g of rye grain spawn and incubated at 25 °C until fully colonized. Spawn run compost was placed aseptically in one half of a sterile 90 mm diam glass Petri dish and sterile casing soil was applied to the other half. The Petri dish was placed in a plastic bag and incubated for 2 - 3 d by which time the mycelium had begun to colonize the casing soil. The Petri dish was removed from the plastic bag and a 1 ml suspension of bacteria was applied to casing soil. The Petri dish was re-incubated at 25 °C in a growth chamber into which fresh air was pumped (50 ml min^{-1}). The humidity within the growth chamber was maintained at ca. 90 %.

A simple system for determining the effect of bacteria on hyphal growth

A. bisporus does not produce primordia when cultured on CMM in association with basidiome inducing bacteria. Nevertheless, valuable information on the effect of bacteria on hyphal growth can be gained by co-culturing bacteria and *A. bisporus* on CMM. The method used was identical to that described above, for achieving fruit body formation of W19 on CMM, except the

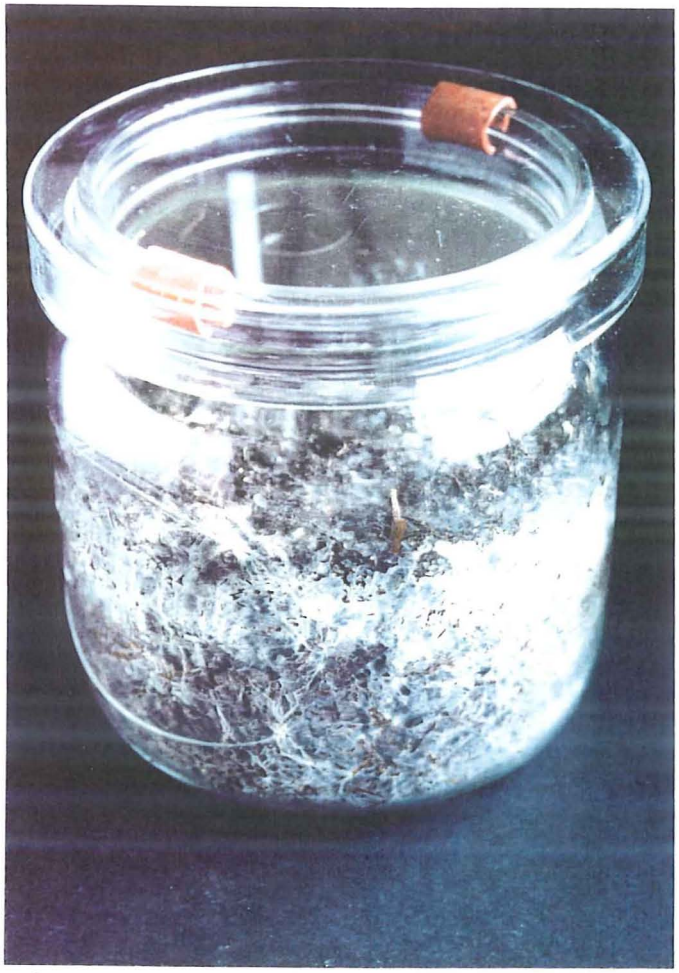


Fig. 4.1. Modified Halbschalentest for the production of *A. bisporus* basidiomata *in vitro*.

temperature of incubation was lowered to 25 °C and the plates were inoculated with bacteria after 5 d growth.

Determination of the suitability of W19 to function as a model strain

The suitability of W19 to function as a model strain for studying the effect of bacteria on basidiome initiation was determined by examining the effect of a range of fluorescent *Pseudomonas* isolates (described in Chapter 2.0.) on basidiome initiation of both W19 and *A. bisporus*. The modified Halbschalentest (described above) was used to achieved basidiome initiation of *A. bisporus*. In addition, the *Pseudomonas* isolates were also cultured alongside *A. bisporus* growing on CMM to determine if any correlation existed between the effect of bacteria on hyphal growth and basidiome initiation.

4.2.4. INVESTIGATIONS INTO THE NATURE OF THE MICROBIAL STIMULUS EFFECTING BASIDIOME INITIATION OF *A. BISPORUS*

A range of experiments were conducted in order to gain further information on the nature of the microbial stimulus effecting basidiome initiation of *A. bisporus*.

The effect of activated charcoal on mycelial growth and basidiome initiation

The effect of activated charcoal on basidiome initiation

The Halbschalentest was used to investigate the effect of activated charcoal on basidiome initiation. The casing layer of each Halbschalentest was supplemented with 5 g of Norit 'Ultrasorb' activated carbon (2.5 g mesh 18 - 52 and 2.5 g mesh 120 - 240), which had been placed in the oven for 4 h at 80 °C and autoclaved (120 °C, 15 min, 1.2 atm) prior to use.

The effect of activated charcoal on hyphal growth

The effect of activated charcoal on hyphal growth was examined by incorporating the activated carbon into CMM prior to autoclaving. Three different concentrations of Norit 'Ultrasorb' activated carbon (mesh 120 - 240) were used; 4, 12 and 24 mg ml^{-1}. Petri dishes containing 25 ml of CMM/activated charcoal were inoculated with a 3 x 3 mm plug of *A. bisporus* taken from

the margin of a 10 d culture. Plates were incubated at 25 °C and colony diameter (the average of two perpendicular measurements) measured at 48 h intervals.

Use of the CMM based bioassays to determine the effect of activated charcoal on hyphal growth and basidiome initiation.

Small patches (20 x 25 mm) of sterile activated charcoal were applied directly to the surface of CMM (in place of bacterial colonies) which had been previously inoculated with either *A. bitorquis* W19 or *A. bisporus*. Activated charcoal was also incorporated into sterile peat (approximately 1:1 by weight) and placed on the surface of the medium alongside W19. A series of controls were used; sterile peat, *P. putida* PMS118S, and sterile peat amended with *P. putida* PMS118S (0.05 ml of washed cells in sterile distiled water; 1 x 10^8 cells ml^{-1}). Three replicates (three bacterial streaks per plate) were used per treatment.

Blocks of CMM in which activated charcoal had been incorporated were also placed alongside the mycelium. This was done by removing 20 x 25 mm blocks of CMM from Petri dishes inoculated with *A. bisporus* and replacing them with similar sized blocks of CMM which were impregnated with activated charcoal (24 mg ml^{-1}).

The effect of iron and chelating agents on mycelial growth and basidiome initiation

Stock solutions of ethylenediaminetetra-acetic acid (EDTA) and $FeCl_3$ were filter sterilized and aliquots of each added aseptically to cooled (50 °C), sterile CMM to give a range of EDTA and $FeCl_3$ concentrations; 0.0, 0.2, 2.0, 20 and 200 μg ml^{-1}. The molten media was poured into 90 mm disposable Petri dishes and the plates centrally inoculated with a 5 mm plug of *A. bisporus* mycelium removed from the growing margin of a 10 d culture. Plates were incubated at 25 °C and colony diameter (the average of two perpendicular measurements) measured every 48 h.

To determine whether EDTA or $FeCl_3$ affected the response of the mycelium to basidiome stimulatory, or inhibitory bacteria, an additional plate of each treatment was incubated for 5 d and then inoculated, on opposite sides of the dish, with *P. putida* PMS118S and *P. tolaasii* PMS117S.

Ethylenediaminedi-O-hydroxyphenyl-acetic acid (EDDA), an iron chelating compound with a higher affinity for Fe^{3+} than EDTA (EDTA stability constant: $\log_{10}K = 25$; EDDA stability

constant: $\log_{10}K$ = 33.9 (Lindsay 1979)), was also incorporated into CMM. A stock solution of EDDA was filter sterilized and aliquots added to molten CMM to give a range of EDDA concentrations; 0, 0.25, 0.5, 1.0 and 2.0 mg ml^{-1}. A range of basidiomycetes, including *A. bisporus*, were inoculated onto the media, incubated at 25 °C and growth assessed visually after 6 d. Growth of *P. putida* PMS118S was also examined on CMM containing EDDA.

The effect of the P. putida *PMS118S siderophore on mycelial growth*

P. putida PMS118S was grown at 28 °C for 48 h in 100 ml flasks containing 20 ml succinate broth (Meyer & Abdallah 1978). The cells were removed by centrifugation, the pH adjusted to pH 7.4 and the broth filter sterilized and freeze dried. The freeze dried extract was resuspended in 1 ml of sterile water and 100 µl was applied to sterile, 10 mm diam antibiotic assay discs, which were placed at the growing margin of 5 d *A. bisporus* cultures. Controls consisted of uninoculated, freeze dried succinate broth.

Determination of the iron status of CMM

The commonly used siderophore auxotroph, *Arthrobacter flavescens* (JG-9) was plated on unamended CMM. In addition, a Petri dish containing CMM was inoculated with a streak of *P. putida* PMS118S and after 24 h incubation the plate was exposed to short wave length (254 nm) UV light to check for the presence of the fluorescent siderophore pigment produced by this organism on iron deplete media (Chapter 2.0.).

The effect of culture filtrates on mycelial growth and basidiome initiation.

P. putida isolates PMS118S and PMS195 were grown in 50 ml of CMM broth at 28 °C for 3 d in an orbital incubator. The bacterial cells were removed by centrifugation (10 min, 8000 *g*) and the supernatant passed through a 0.22 µm Millipore filter. The culture filtrates were concentrated by freeze drying and the resulting powder dissolved in 2 ml of sterile distilled water. Uninoculated CMM broth was used as a control.

The culture filtrates were applied to sterile 10 mm diam antibiotic assay discs; 0, 50, 100, 250 and 500 µl of filtrate per disc. The effect of the filtrates on mycelial growth and basidiome initiation was determined by placing the discs alongside 5 d cultures of *A. bisporus* and 7 d

cultures of *A. bitorquis* W19, according to the procedures described above (section 4.2.3.). Filtrates were also applied directly to the medium.

The transition from nutrient rich to nutrient poor media and its effect on mycelial growth and basidiome initiation.

Disposable 90 mm Petri dishes with a central division were filled with CMM in one half. The second half was filled with either 1/10 strength CMM, or water agar, both of which were solidified with 1.5 % agar. Control plates contained CMM in both sides. Plates were inoculated, in the centre of the compartment containing CMM, with a 3 x 3 mm plug of *A. bisporus* removed from the growing margin of a 10 d culture. Plates were incubated at 25 °C and the growth of the fungus from nutrient rich, to nutrient poor substrate, was examined. Growth was assessed visually, as a measured growth rate was dependent upon the position of the inoculum and this could not be readily controlled. Five replicates of each treatment were used. In some instances a 20 μl drop containing approximately 1 x 10^6 of washed *P. putida* PMS118S cells was applied to the nutrient poor section of the plate and the response of the mycelium to the bacterium assessed visually.

The response of *A. bisporus* mycelium to a glass surface was also observed. A sterile glass cover slip was placed on the surface of CMM after the fungal colony had reached a diameter of approximately 20 mm. The growth of the mycelium over the nutrient poor zone was observed with the naked eye.

The effect of non-living *P. putida* PMS118S cells on mycelial growth and basidiome initiation.

Incorporation of non-living P. putida *PMS118S cells into CMM.*

P. putida PMS118S was grown in 250 ml flasks containing 100 ml CMM broth. Cultures were incubated overnight in an orbital shaker at 28 °C and cells harvested by centrifugation (10 min, 8000 *g*). The cell pellet from each 250 ml flask was added to 100 ml of fresh CMM and the fresh medium containing *P. putida* PMS118S was autoclaved (120 °C, 15 min, 1.2 atm) and dispensed (25 ml) into disposable 90 mm Petri dishes. In some instances, the cells contained within the

flasks of fresh media were disrupted by ultra-sonication using a 'Megason' ultrasonic disintegrator (50 % power, 8 min) before autoclaving.

P. putida PMS118S was also grown overnight at 28 °C on CMM in 90 mm Petri dishes. Growth from the surface of four Petri dishes was removed by scraping and added to 100 ml of fresh CMM which was treated as described above.

Petri dishes were inoculated with a 3 x 3 mm plug of *A. bisporus* taken from the growing margin of a 10 d culture. Plates were incubated at 25 °C and colony diameter (the average of two perpendicular measurements) measured at 48 h intervals. Control plates consisted of unamended CMM.

Application of non-living P. putida *PMS118S to the surface of CMM.*

P. putida PMS118S was cultured overnight on CMM and KB in 90 mm Petri dishes at 28 °C. Cells were killed either by treating with chloroform or heat.

Chloroform A watchglass containing 0.5 ml chloroform was placed in the lid of an inverted Petri dish and left at room temperature. After 30 min the chloroform was removed and the plate left overnight to ensure all chloroform vapours had dispersed. Cell death was checked by streaking on KB. The mass of dead cells was collected by scraping and stored in a sealed 3 ml glass vial at 4 °C.

Heat Overnight growth of *P. putida* PMS118S was collected from the surface of CMM plates and placed in 3 ml glass vials. These were autoclaved (120 °C, 15 min, 1.2 atm) and stored at 4 °C.

To determined the effect of non-living *P. putida* PMS118S on mycelial growth and basidiome initiation the two bioassays described above (section 4.2.3.) were used. The dead cells were spread thickly on the surface of the medium so as to simulate the density of cells found in a living *P. putida* colony. Dead cells were also incorporated into sterile peat and applied to the surface of CMM. Three replicate plates (three bacterial streaks per plate) were used for each treatment

Hyphal growth experiments

The effect of a lawn of P. putida *PMS118S on hyphal growth of* A. bisporus

A 90 mm diam Petri dish containing CMM was inoculated with a lawn of *P. putida* PMS118S. A 5 mm diam plug of *A. bisporus* mycelium, removed from the growing margin of a 10 d colony, was applied to the centre of the plate. The Petri dish was incubated at 25 °C and colony diameter (the average of two perpendicular measurements) was determined at 48 h intervals. The growth of *A. bisporus* over the lawn of *P. putida* PMS118S was compared with the growth of *A. bisporus* over CMM.

Examination of the ability of mycelium growing in the presence of P. putida *PMS118S to retain the same rapidly extending growth form when sub-cultured and grown in the absence of the bacterium*

A. bisporus mycelium was grown on CMM in association with *P. putida* PMS118S as described above (section 4.2.3.). A small piece of rapidly extending mycelium was removed from above a *P. putida* PMS118S colony and sub-cultured onto a fresh plate of CMM and grown in the absence of the bacterium. An equally small piece of mycelium, of similar age, but growing in the absence of the bacterium, was removed from the same plate and also sub-cultured onto a fresh plate of CMM. The radial growth rate of the two cultures was compared by measuring colony diameter every 48 h. This experiment was repeated five times.

4.2.5. THE EFFECT OF *P. PUTIDA* PMS118S ON HYPHAL GROWTH OF *A. BISPORUS*

Interaction between *P. putida* PMS118S and *A. bisporus*

The effect of *P. putida* PMS118S on hyphal growth was examined by co-culturing the bacterium with *A. bisporus* on CMM as follows. CMM (25 ml) was poured into 90 mm disposable Petri dishes and left overnight to dry before centrally inoculating with a 3 x 3 mm plug of *A. bisporus* removed from the growing margin of a 10 d culture. After 6 d incubation at 25 °C, *P. putida* PMS118S was inoculated onto the surface of the medium and spread with either a platinum wire loop or a small glass spreader, to cover (unless otherwise stated), a rectangular area, 20 x 25 mm, as shown in Fig. 4.2. The Petri dishes were re-incubated at 25 °C and the mycelium

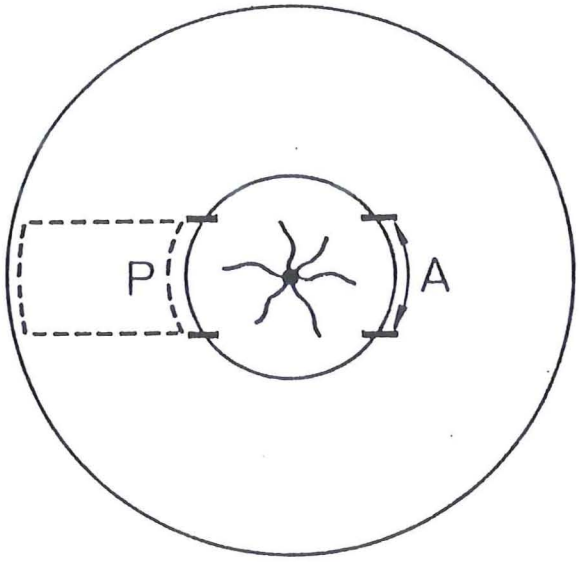


Fig. 4.2. Petri dish system for examining the effect of *P. putida* PMS118S on hyphal growth of *A. bisporus.* Dotted lines show the outline of the bacterial colony and heavy lines denote 20 mm wide region of the colony margin where measurements of hyphal growth were taken.
P, presence of bacteria; **A**, absence of bacteria

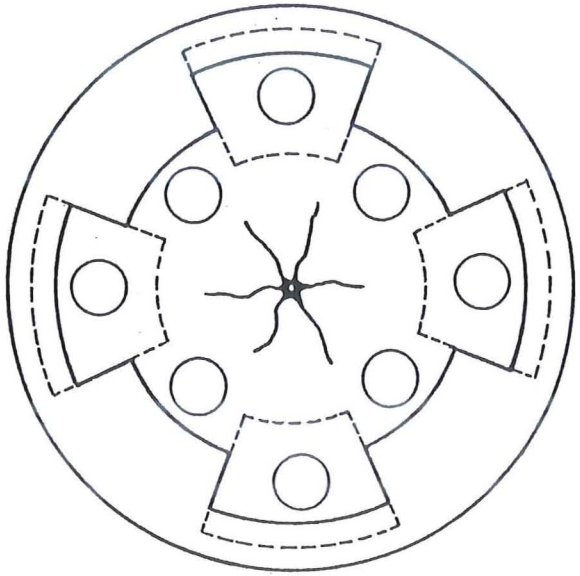


Fig. 4.3. Diagrammatic representation of an *A. bisporus* colony at the time of dry weight of fungal tissue determination. Dotted lines show outline of the bacterial colony and solid lines denote margin of the fungal colony. Circles illustrate the location of 10 mm diam plugs of agar and mycelium which were removed for determination of dry weight of fungal tissue.

allowed to grow over the surface of the bacterial colony. Control plates were not inoculated with bacteria.

In Petri dishes containing both *A. bisporus* and *P. putida* PMS118S, hyphal growth was measured in the *presence* and *absence* of bacteria. Measurements were confined to 20 mm wide sections of the colony margin and the same sections were measured over consecutive time intervals (Fig. 4.2). Hyphal growth was also measured within a 20 mm wide section of the colony margin on control plates.

Measurement of mycelial extension, hyphal diameter and branch-angle

The rate of mycelial growth was determined by measuring the distance from a mark scored on the underside of the Petri dish to the colony margin. The same 20 mm wide section of the colony margin was measured on consecutive days by placing the entire Petri dish, with lid, under a light microscope equipped with an ocular micrometer. The diameter of leading hyphae and the branch-angle were determined from photomicrographs which were projected on to a screen.

Measurement of mycelial branching

For technical reasons (density of growth) it was not possible to measure mycelial branching using methods such as the 'hyphal growth unit' (HGU) (Trinci 1974), or the 'internode length unit' (ILU) (Armentrout *et al.* 1986). Instead a modification of the ILU was used, the 'subapical internode length' (SIL). The SIL was defined as the mean length of ten subapical internodes (distance between the apical and subapical branch junctions) chosen at random within a predetermined, 20 mm section of the colony margin (Fig. 4.2). The SIL was determined by placing the entire Petri dish, with lid, under a light microscope equipped with an ocular micrometer and the SIL of leading hyphae from the same section of the colony margin was determined on consecutive days.

Measurement of mycelial dry weight

The effect of *P. putida* PMS118S on the dry weight of *A. bisporus* mycelium was determined using a modification of the procedure described by Sutton & Starzyk (1972). Four plugs of agar and mycelium, 10 mm diam, were removed with a cork borer from predetermined sections of

each Petri dish (*presence* or *absence* of bacteria) as shown in Fig. 4.3. The agar-mycelial plugs were removed from 3 mm behind the colony margin, sampling mycelium which ranged from 1 to 4 d. The four plugs from each section of each Petri dish, were pooled and placed in 50 ml of rapidly boiling water for 1 min. They were then transferred to a beaker containing 200 ml of boiling water and left for a further 2 min before placing on a pre-weighed section of Whatman no. 1 filter paper. The filter paper plus mycelial discs were dried at 70 °C for 24 h and transferred to a desiccator before weighing. Determination of the dry weight of mycelium from entire plates, or large sections of plates, was not possible, as the boiling water treatment did not completely remove agar from these regions.

It was conceivable that the effect of *P. putida* PMS118S on the production of fungal material was dependent on the area occupied by the bacterium. Petri dishes were therefore inoculated with *P. putida* PMS118S so as (i) the entire area of the Petri dish not colonized by the 6 d fungal colony, was covered with the bacterium, (ii) half of this area (Fig. 4.3) and (iii) one quarter of this area was covered with the bacterium. Control plates were not inoculated with the bacterium (see Fig. 4.28).

4.2.6. STATISTICAL ANALYSIS

Statistical analysis was performed using standard ANOVA techniques. Comparisons between means were made using the least significant difference test (L.S.D.).

Planned comparisons of control versus individual treatments were made using Bonferroni's pairwise comparisons to allow for multiple comparisons.

4.3. RESULTS

4.3.1. GROWTH OF *A. BISPORUS* ON CMM

CMM promoted rapid, sustained growth of vegetative mushroom mycelium, enabling complete colonization of a 90 mm diam Petri dish to occur within 2 wk (Fig. 4.4). On media other than CMM, the rate of linear extension of hyphae decreased once the Petri dish was approximately 50 % colonized (10 - 14 d). On CEA this decline in radial growth rate was not marked, but on 2 % MEA-Bacto the rate of hyphal extension decreased to such an extent that 8 - 10 weeks was required for *A. bisporus* to fully colonize a 90 mm diam Petri dish. On 2 % MEA-Maltexo, complete colonization of the Petri dish took three and a half weeks which is comparable to the growth of *A. bisporus* on 2 % MEA (Boots Pure Drug Co., U.K.) reported by Wood (1976).

The vigour of the growth stimulated by CMM is shown in Fig. 4.5. Growth of *A. bisporus* was markedly affected by different media:- PDA and MPA both stimulated dense, uneven growth in which sectoring was apparent, 2 % MEA-Bacto promoted weak, uneven growth (Fig. 4.5) and the basal medium stimulated slow growth in which sectoring regions were common. On 2 % MEA-Maltexo and on CEA the growth of mushroom mycelium was even and few sectoring areas were seen, however, on CEA growth was sparse. Sectoring was rarely observed on CMM and growth was extremely vigorous.

Comments on the effect of CMM components on the growth of *A. bisporus*

The growth of *A. bisporus* was markedly affected by the source of malt extract. The Oxoid product was the most suitable, other brands including, Bacto and BBL, encouraged uneven, sectored growth. The concentration of malt extract had little effect on the radial growth rate, but as the concentration was increased, from 0.25 % to 4.0 %, a corresponding increase in the density of hyphae at the colony margin was observed.

CMM was made from composts obtained both from local mushroom growers and from I.H.R. and the effect of these different composts on the performance of CMM was negligible.

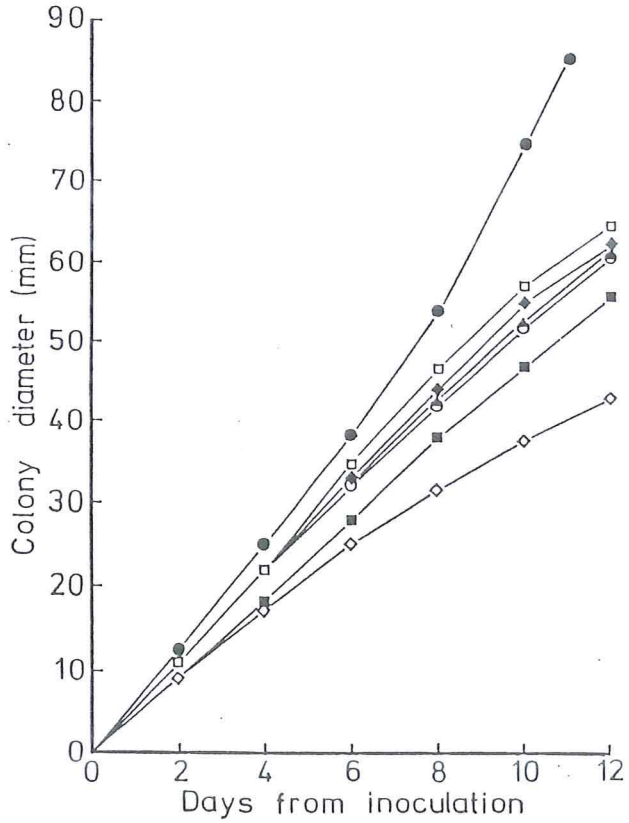


Fig. 4.4. Colony growth of *A. bisporus* on; CMM (●), MEA-Maltexo (□), PDA (◆), CEA (▲), MPA (○), basal medium (■), and MEA-Bacto (◇). Data are means of five replicates. S.E. are contained within the symbols.

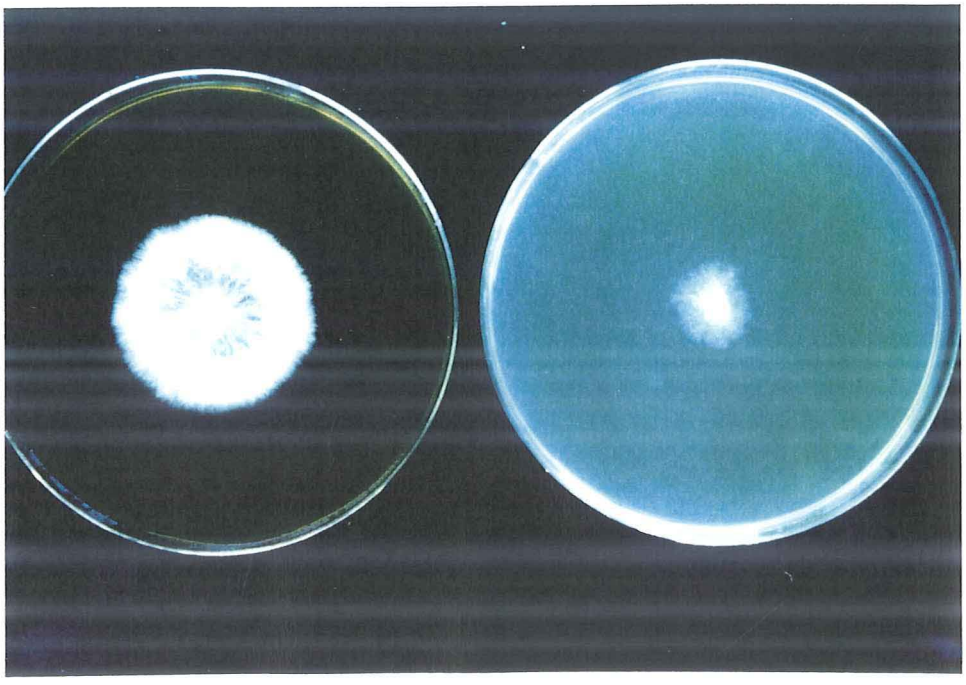


Fig. 4.5. Growth of *A. bisporus* on CMM and MEA-Bacto, 6 d after inoculation.

The method by which the compost was infused affected the growth of *A. bisporus*, as did the strength of the infusate. Significantly more concentrated infusates (1.5 - 2.0 times stronger) resulted in slower, denser, slightly sectored growth.

Infusate collection through muslin cloth allowed a significant amount of insoluble, particulate material to remain in the infusate which was beneficial to the growth of *A. bisporus*. Removal of this fraction by filtration resulted in growth which was less vigorous. The addition of nitrogen (0.01 - 0.1%) as mineral N, or urea, decreased the radial growth rate. Addition of 10 mM phosphate buffer had no observable effect on the growth of *A. bisporus*.

CMM stimulated good growth in basidiomycetes other than *A. bisporus*, including, *A. bitorquis*, *A. campestris*, *Auricularia auricula*, *Coprinus bilanatus*, *Flammulina velutipes*, *Lentinus edodes*, *Pleurotus ostreatus*, *P. flabelatus* and *Volvariella volvacea*. CMM from which agar was omitted also served as a good growth medium for *A. bisporus*.

4.3.2. *IN VITRO* FRUITING OF *A. BISPORUS*: A MODEL SYSTEM

Assessment of the suitability of W19 to function as a model strain for examining the involvement of bacteria in basidiome initiation of *A. bisporus*.

The effect of fifteen selected *Pseudomonas* isolates on basidiome initiation of *A. bisporus* and *A. bitorquis* (W19) is shown in Table 4.1. *Pseudomonas* isolates had the same effect on basidiome initiation of W19 on CMM, as they did on fruiting of *A. bisporus* in the modified Halbschalentest.

Fruiting of W19 on CMM

W19 formed basidiome initials in association with hyphal strands (Fig. 4.6) and these appeared as fluffy, white mycelial aggregates (1 mm diam), 4 - 7 d after inoculation with bacteria and developed into smooth white primordia (2 - 5 mm diam), after 2 - 3 d (Figs 4.7 and 4.8). The number of basidiome initials produced frequently exceeded eight and in these instances crowding caused the primordia to clump, making it impossible to distinguish individual fruit bodies (Fig. 4.7). Basidiome initials were formed directly above the bacterial colonies and radial growth continued during the process of initiation. The rate of hyphal extension was promoted by

basidiome stimulatory pseudomonads prior to the appearance of primordia and mycelial strands were usually more pronounced and numerous over the bacterial colonies. Primordia failed to develop beyond a diameter of 8 mm, but examination of these structures by light microscopy revealed them to be analogous to normal primordia (Fig. 4.9). Basidiome initials were detected only in association with basidiome stimulatory bacteria and primordia were produced in all replicate plates. W19 cultured on CMM, in the manner described above, but in the absence of basidiome stimulatory bacteria, failed to initiate fruit body development.

Table 4.1. The effect of selected *Pseudomonas* isolates on basidiome initiation of *A. bitorquis* W19 on CMM and *A. bisporus* in Halbschalentests, and on mycelial growth of *A. bisporus* (U3) on CMM.

		BASIDIOME INITIATION		HYPHAL GROWTH
Bacterial isolate		**W19 on CMM[a]**	**U3 in Halb-schalentests[b]**	**U3 on CMM[c]**
Control		0[d]	1.7 + 1.9[e]	0[f]
P. putida	PMS118S	24	52.7 + 12.6*	+
P. putida	PMS195	24	34.8 + 8.7*	+
P. puitda	PMS196	24	39.5 + 10.9*	+
P. putida	PMS234	24	42.2 + 11.0*	+
P. putida	PMS233	24	46.3 + 15.3*	+
Pseudomonas sp.	PMS220	24	43.3 + 13.8*	+
Pseudomonas sp.	PMS132	24	32.0 + 8.2*	+
P. fluorescens	PMS157	0	1.2 + 1.7	0
P. fluorescens	PMS382	0	0	-
P. tolaasii	PMS117	0	0	-
'P. reactans'	PMS273	0	0	-
P. agarici	PDDCC2656	0	0	-
Pseudomonas sp.	PMS126	0	1.0 + 1.5	-
Pseudomonas sp.	PMS127	0	0	-
Pseudomonas sp.	PMS129	0	0	-

[a,c] Eight replicates (three bacterial streaks per plate).
[b] Six replicates
[d] The number of bacterial streaks above which primordia were initiated- no bacteria were present on control plates.
[e] Mean number of primordia initiated per jar (+ S.D.)
[f] +, stimulation; 0, no effect; -, inhibition.
* Number of primordia is greater than control ($P < 0.05$).

Occasionally cultures of W19 formed primordial initials in the absence of *P. putida* in Petri dishes which had been removed from the 30 °C incubator and left at room temperature for 2 - 3

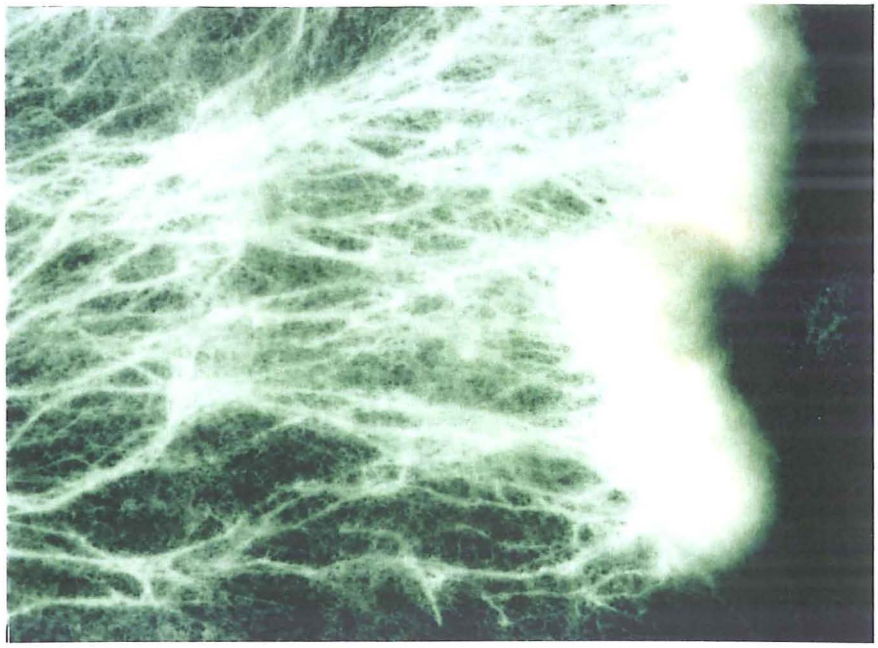


Fig. 4.6. The formation of primordia by *A. bitorquis* W19 in association with mycelial strands on CMM (x 3.5).

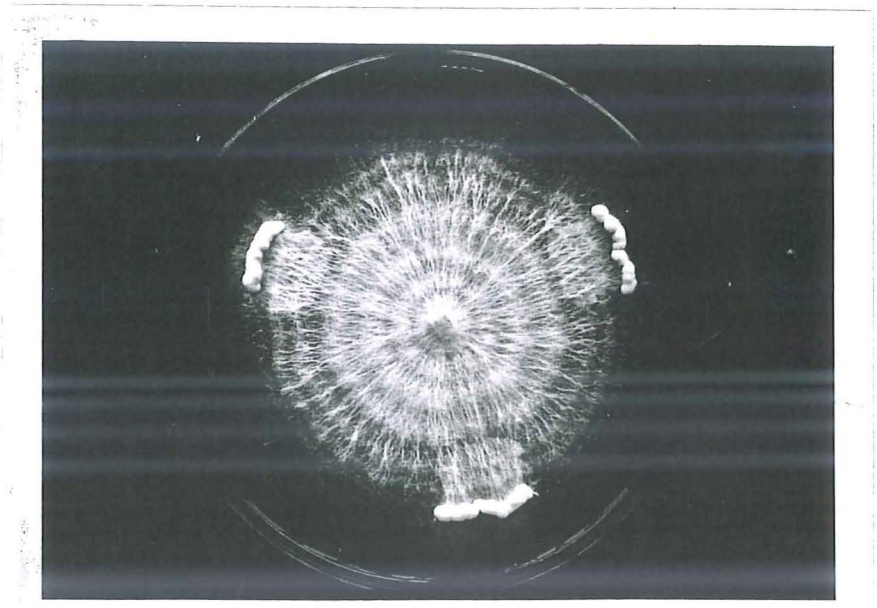


Fig. 4.7. The effect of *P. putida* PMS118S on basidiome initiation of *A. bitorquis* W19.

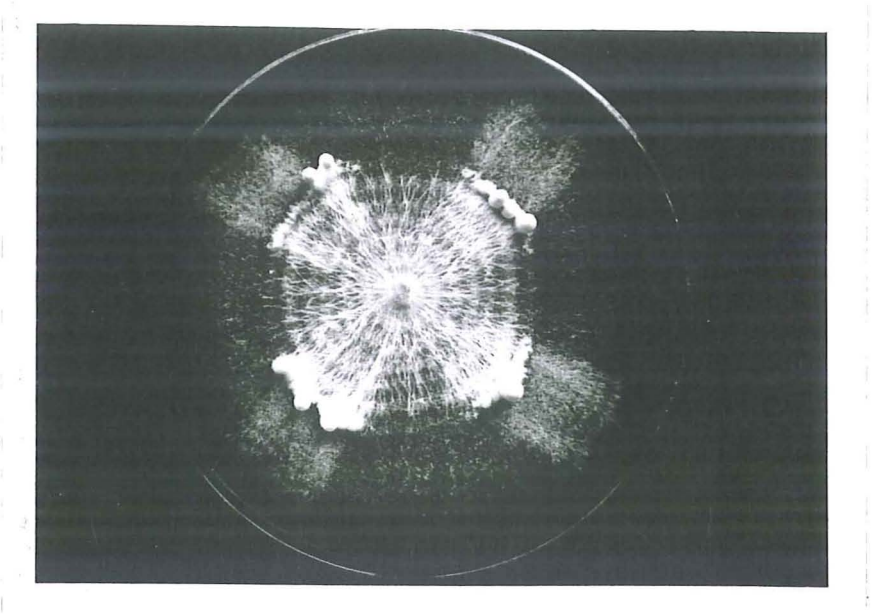


Fig. 4.8. The effect of *P. putida* PMS234 on basidiome initiation of *A. bitorquis* W19.

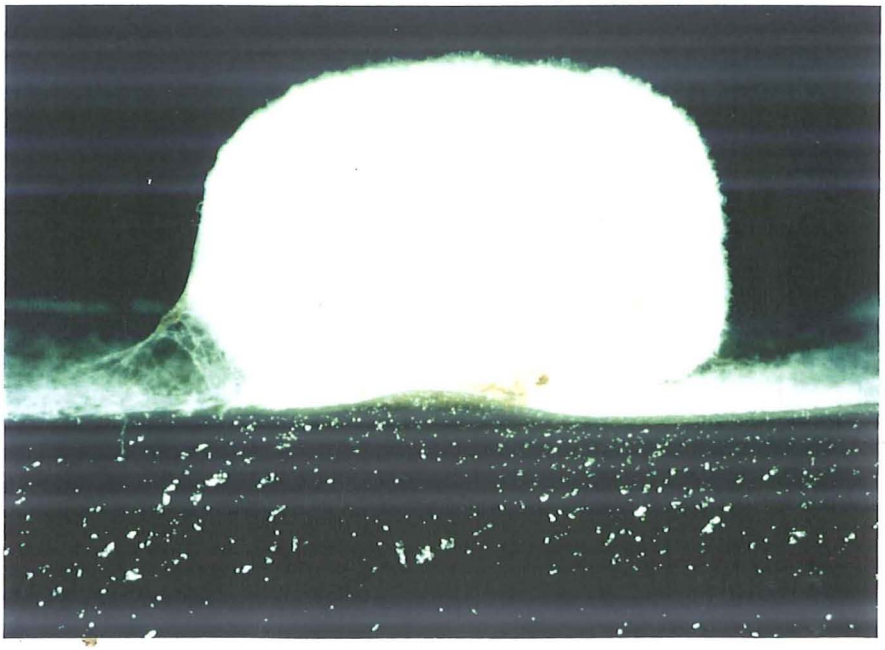


Fig. 4.9. A section through an *A. bitorquis* W19 primordium (7 mm diam) formed on CMM in association with *P. putida* PMS118S, in which the initial stages of fruit body differentiation are discernible.

wk (18 - 22 °C). Under these conditions, the initials resembled mycelial knots, remained small (1 - 2 mm diam), irregular and 'fluffy' in appearance (Fig. 4.10). These initials were produced randomly near the colony margin and did not form in association with mycelial strands. Prolonged incubation of W19 (up to 8 wk) at 30 °C in the absence of basidiome stimulatory bacteria, did not stimulate mycelial knot formation.

Fruiting of A. bisporus *in the modified Halbschalentest*

Primordia produced in the modified Halbschalentests appeared within 10 d of inoculation with bacteria and an average of five per jar developed into mature basidiomata capable of liberating spores. Basidiome initials occasionally developed in the absence of bacteria, but their appearance was delayed 1 - 2 wk and the number produced was considerably less than the number produced in the presence of basidiome stimulatory bacteria (Table 4.1).

The relationship between the response of *A. bisporus* hyphae to bacteria on CMM and fruiting of *A. bisporus* in the modified Halbschalentest

The effect of fifteen selected *Pseudomonas* isolates on basidiome initiation of *A. bisporus* in the modified Halbschalentest and hyphal growth of *A. bisporus* on CMM is shown in Table 4.1.

Pseudomonads which promoted basidiome initiation of *A. bisporus* in the modified Halbschalentest caused the rate of hyphal extension of *A. bisporus* mycelium to increase markedly in the vicinity of the bacterial colonies (Fig. 4.11). Isolates which failed to promote basidiome initiation of W19, or *A. bisporus* in the modified Halbschalentest, did not stimulate hyphal growth of *A. bisporus* on CMM (Table 4.1). *A. bisporus* did not produce basidiome initials on CMM under any conditions.

Use of the model system to determine of the effect of a range of bacteria on basidiome initiation and hyphal growth of *A. bisporus*.

A preliminary investigation was made into the effect of a range of bacteria, including non-casing layer derived isolates, on basidiome initiation and hyphal growth (Table 4.2, see also Table 4.1). The model system provided a means of determining the effect of pathogenic and saprophytic pseudomonads on mycelial growth. The toxin producing isolate of *P. tolaasii* PMS117S markedly

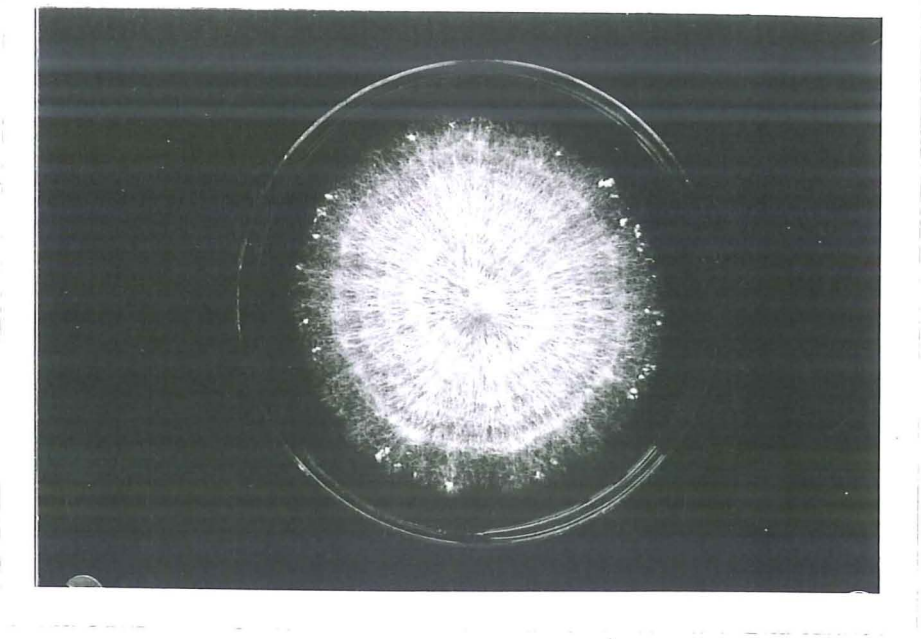


Fig. 4.10. The formation of mycelial knots by *A. bitorquis* W19 on CMM in the absence of basidiome stimulatory bacteria.

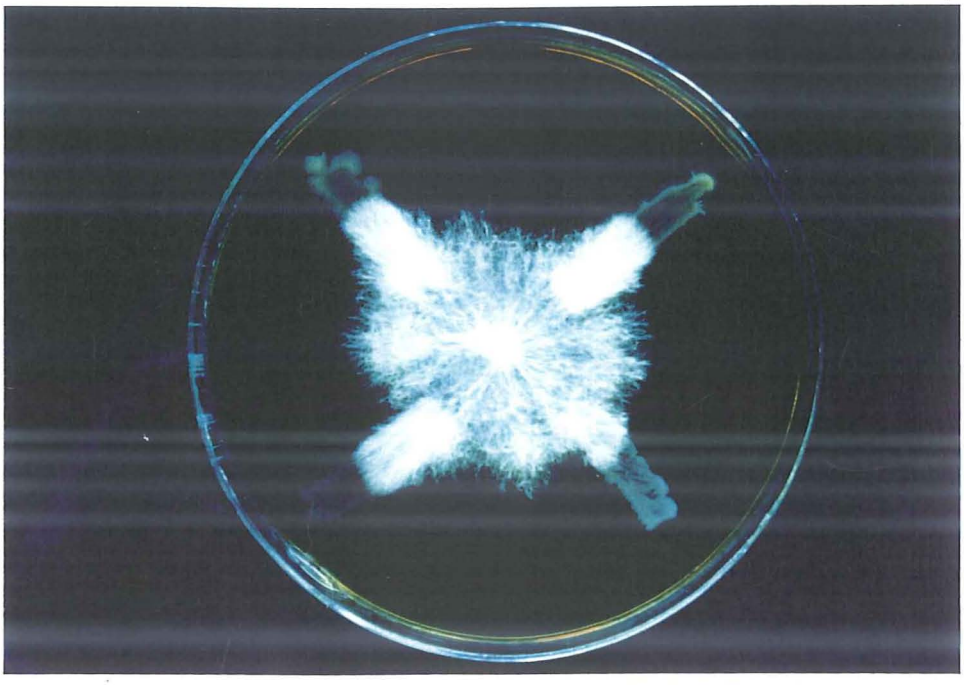


Fig. 4.11. The effect of three basidiome stimulatory isolates of *P. putida* PMS118S (top RH), PMS195 (top LH), PMS234 (bottom LH) and a non-basidiome stimulatory *P. fluorescens* PMS157 (bottom RH) isolate, on mycelial growth of *A. bisporus* on CMM.

Table 4.2. The effect of a range bacteria on basidiome initiation of the model strain *A. bitorquis* W19, and hyphal growth of *A. bisporus*.

Bacterial isolate	Source	Basidiome Initiation[a]	Hyphal growth[a]
Bacillus subtilis	Microbiology University of Canterbury	-[b] lysis	-[c]
Erwinia amylovora	HKM, Genetics,	-	-
E. herbicola	University of	-	-
Escherichia coli	Canterbury		
K12		-	-
Rhizobium sp.	NMU, Genetics, University of Canterbury	-	-
Serratia marsecens			
UC6	HKM	-	-
UC9	HKM	-	-
UC7	HKM	-	-
P. aeruginosa			
OT11	Microbiology Dept	-	0
PAO5	Otago University	-	-
P. syringae	DSIR,		
PDDCC7607	Auckland	-	--
'P. gingeri'	DSIR		
PDDCC8872	Auckland	-	-
'P. gingeri'	Mushroom cap		
PMS140	This study	-	--
Pseudomonas sp.	Mushroom cap		
PMS119	This study	-	-
Pseudomonas sp.	Mushroom cap		
PMS120	This study	- lysis	-
Pseudomonas sp.	Mushroom cap		
PMS211	This study	-	-
Pseudomonas sp.	Casing layer		
PMS141	This study	+	+
Pseudomonas sp.	Casing layer		
PMS142	This study	+	+
Pseudomonas sp.	Casing layer		
PMS143	This study	+	+
Non fluorescent sp.	Casing layer		
PMS124	This study	- lysis	--
Non fluorescent sp.	Casing layer		
PMS125	This study	- lysis	-
Non fluorescent sp.	Casing layer		
PMS132	This study	- lysis	-

[a]One plate (four bacterial streaks per plate)
[b]+, primordia; -, no primordia
[c]+, stimulation; 0, no effect; -, inhibition; --, strong inhibition
lysis; lysis of bacterial colony

inhibited mycelial growth (Fig. 4.12) as did *P. fluorescens* PMS382. Mycelial growth was also inhibited by *P. agarici* PDDCC2656 and '*P. gingeri*' PDDCC8872, although in these instances inhibition did not occur until hyphal contact was made with the bacterial colony. Growth of hyphae over colonies of '*P. reactans*' NCPB3149 and PMS273 occurred, but the rate of hyphal extension decreased and strand formation was inhibited. *P. fluorescens* PMS157 had no effect on the rate of mycelial growth of *A. bisporus*, but displayed slight inhibition of W19.

Some isolates, including one belonging to the genus *Pseudomonas*, were lysed by the mushroom mycelium. Colony lysis was detectable with the naked eye and was evident as a cleared region in the bacterial colony which preceded the advancing mycelium (Fig. 4.13). Approximately 50 unidentified non-fluorescent bacteria, isolated from the casing layer, were screened against W19. None of these isolates were able to promote basidiome initiation and the result of only three such interactions are recorded in Table 4.2. More than half of the non-fluorescent isolates were lysed by the advancing mycelium and examination of bacteria removed from the cleared zone with a light microscope revealed the presence of distorted bacterial cells.

4.3.3. INVESTIGATIONS INTO THE NATURE OF THE MICROBIAL STIMULUS EFFECTING BASIDIOME INITIATION OF *A. BISPORUS*

The effect of activated charcoal on hyphal growth and basidiome initiation

The effect of activated charcoal on basidiome initiation

The effect of activated charcoal on basidiome initiation of *A. bisporus* in halbschalentests is shown in Table 4.3. Activated charcoal promoted basidiome initiation when incorporated into sterile peat casing soil. When compared to both non-sterile peat, and sterile peat supplemented with *P. putida*, peat amended with activated charcoal promoted fewer primordia which took longer to appear.

A. bitorquis W19 did not form primordia on CMM when cultured in the presence of activated charcoal (Fig. 4.14), however, primordia were produced if the activated charcoal was incorporated into sterile peat. Sterile peat failed to promote basidiome initiation, but peat supplemented with *P. putida* PMS118S, and *P. putida* PMS118S applied directly to the surface of

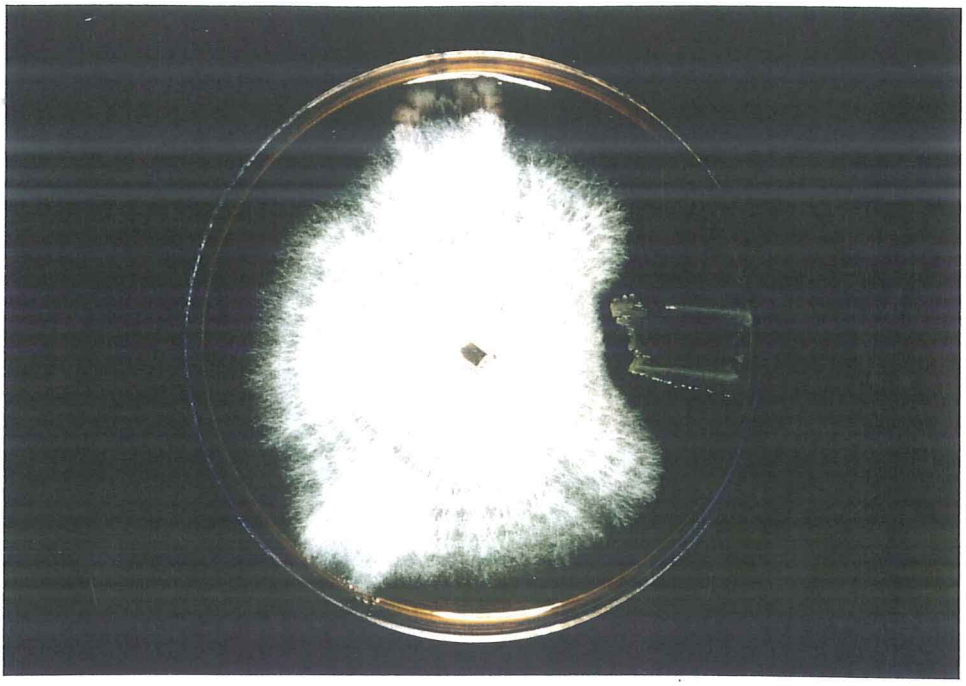


Fig. 4.12. The effect of *P. putida* PMS118S (bottom) and PMS118R (top) and *P. tolaasii* PMS117S (right) on mycelial growth and basidiome initiation of *A. bitorquis* W19. Primordia are just visible over PMS118S and PMS118R colonies.

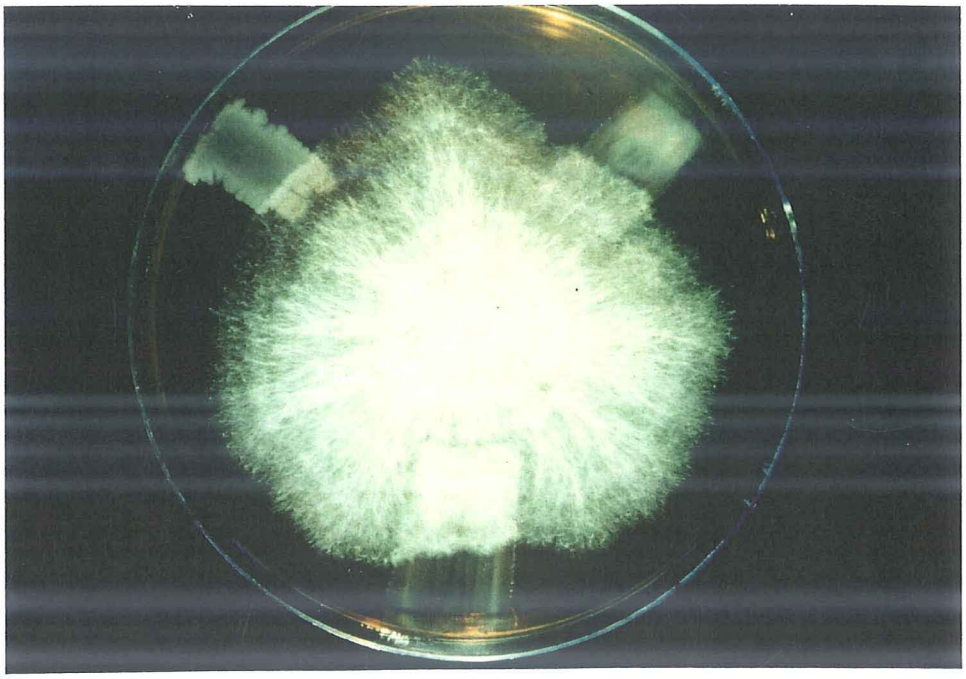


Fig. 4.13. The effect of *Pseudomonas* isolates PMS211 (left), PMS120 (right) and PMS119 (bottom) on mycelial growth and basidiome initiation. Note clearing (lysis) of PMS120.

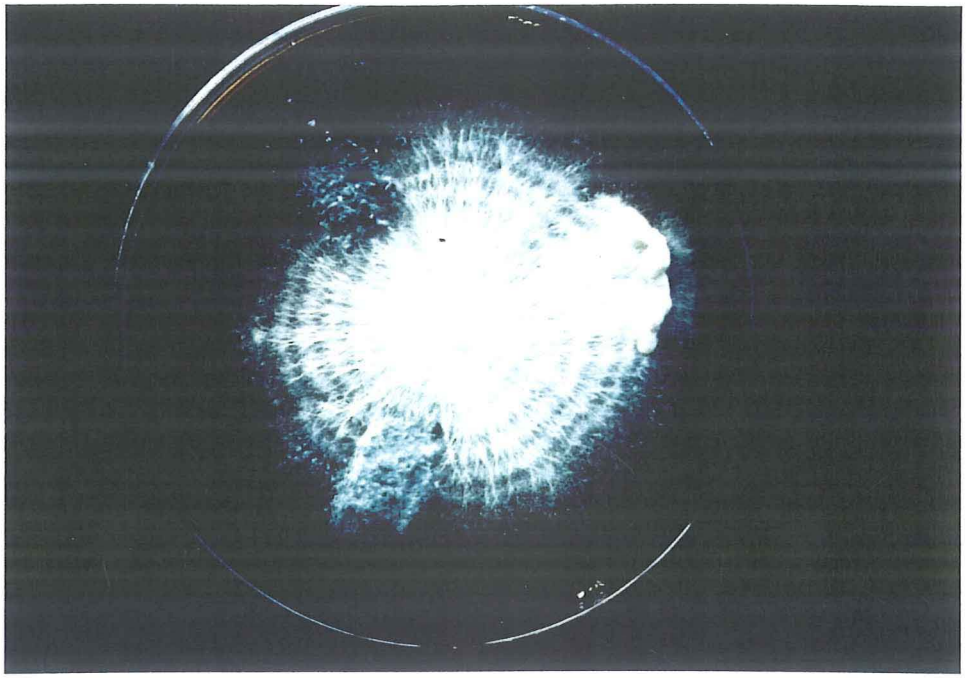


Fig. 4.14. The effect of activated charcoal on basidiome initiation of *A. bitorquis* W19. Fine mesh (bottom), coarse mesh (top), *P. putida* PMS118S (right).

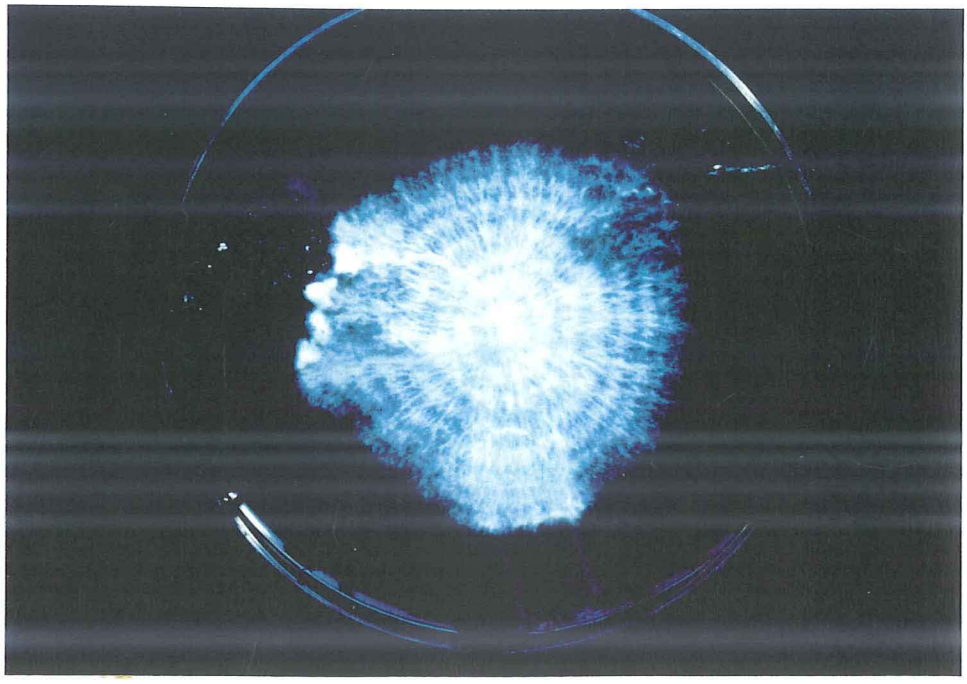


Fig. 4.15. The effect of sterile peat (right), sterile peat supplemented with *P. putida* PMS118S (left) and *P. putida* PMS118S (bottom) on basidiome initiation of *A. bitorquis* W19. Primordia are beginning to develop over the PMS118S colony - note the thickening of hyphae at the colony margin.

the medium, both promoted basidiome initiation (Fig. 4.15). Basidiome initiation was frequently more rapid and the resulting primordia often grew to a larger size (8 - 10 mm diam) when *P. putida* was incorporated into peat.

Table 4.3. The effect of activated charcoal on basidiome initiation of *A. bisporus*

Treatment	Number of primordia[a]
Sterile casing	0
Sterile casing plus activated charcoal	15.3 + 10.3*
Sterile casing plus *P. putida* PMS118S	27.0 + 8.1*
Non-sterile casing	25.2 + 11.2*

Primordia were produced *in vitro* using the Halbschalentest.
Data are means + SD of six replicates.
[a]Primordia were defined as smooth mycelial aggregates greater than 1 mm diameter.
*Significantly greater than control ($P < 0.05$)

The effect of activated charcoal on hyphal growth

Activated charcoal inhibited the rate of radial growth of *A. bisporus* mycelium when it was incorporated into CMM and the degree of inhibition was proportional to the concentration of activated charcoal (Fig. 4.16). When activated charcoal was present in only small patches on the surface of CMM the rate of hyphal extension over these areas was increased (Fig. 4.17). This response was similar to the effect of basidiome stimulatory pseudomonads on hyphal growth of *A. bisporus* on CMM.

The effect of iron and chelating agents on mycelial growth and basidiome initiation

The effect of $FeCl_3$ and EDTA on colony diameter is shown in Figs 4.18 and 4.19. No primordia were detected on any Petri dish, at any $FeCl_3$, or EDTA concentration. Mycelial growth had a tendency to be 'fluffy' in the presence of $FeCl_3$.

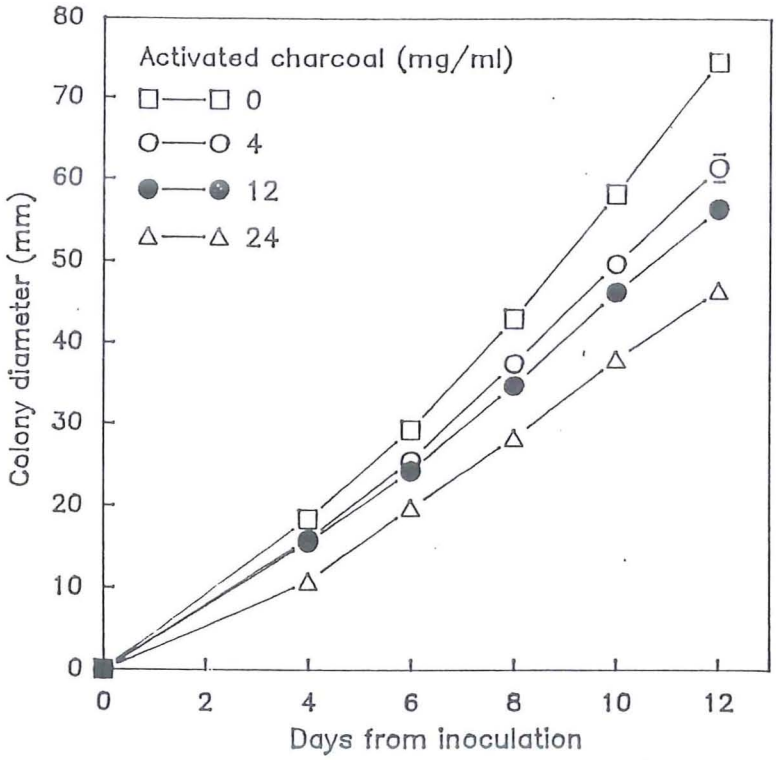


Fig. 4.16. The effect of activated charcoal on hyphal growth of *A. bisporus*. Data are means of four replicates and S.E. are shown when they exceed the diameter of the symbols.

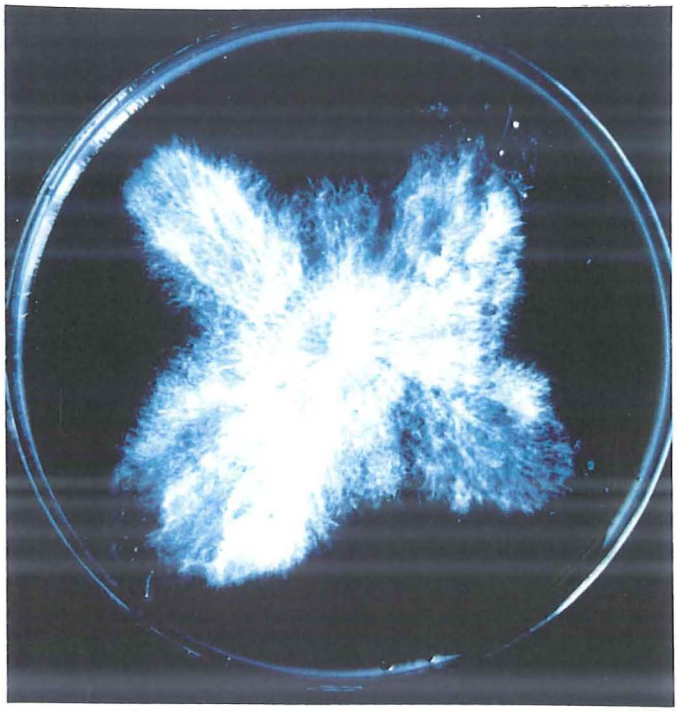


Fig. 4.17. The effect of activated charcoal and peat on mycelial growth of *A. bisporus*. Coarse mesh (top LH); fine mesh (bottom RH). Sterile peat (top RH), sterile peat supplemented with *P. putida* PMS118S (bottom LH).

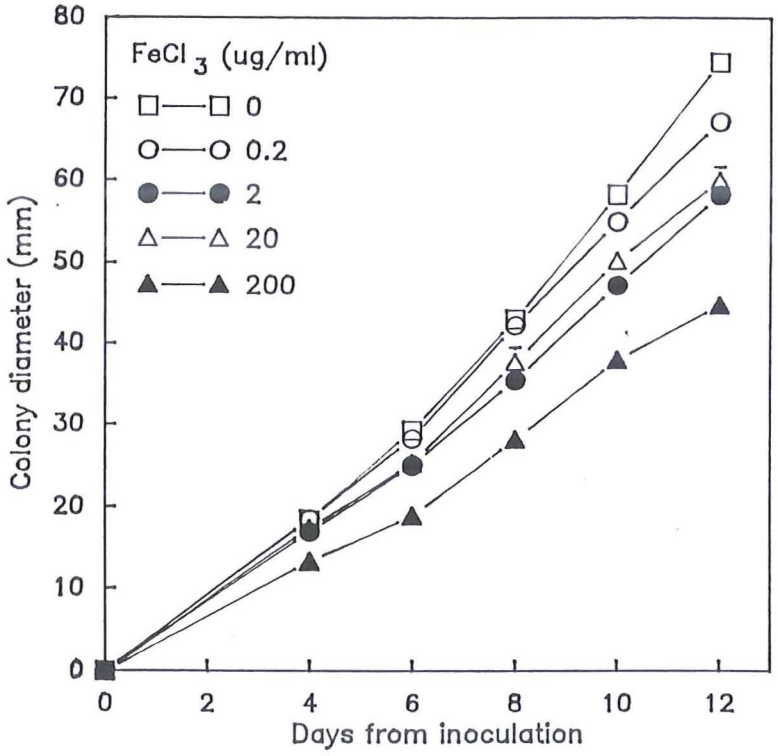


Fig. 4.18. The effect of $FeCl_3$ on hyphal growth of *A. bisporus*. Data are means of four replicates and S.E. are shown when they exceed the diameter of the symbols.

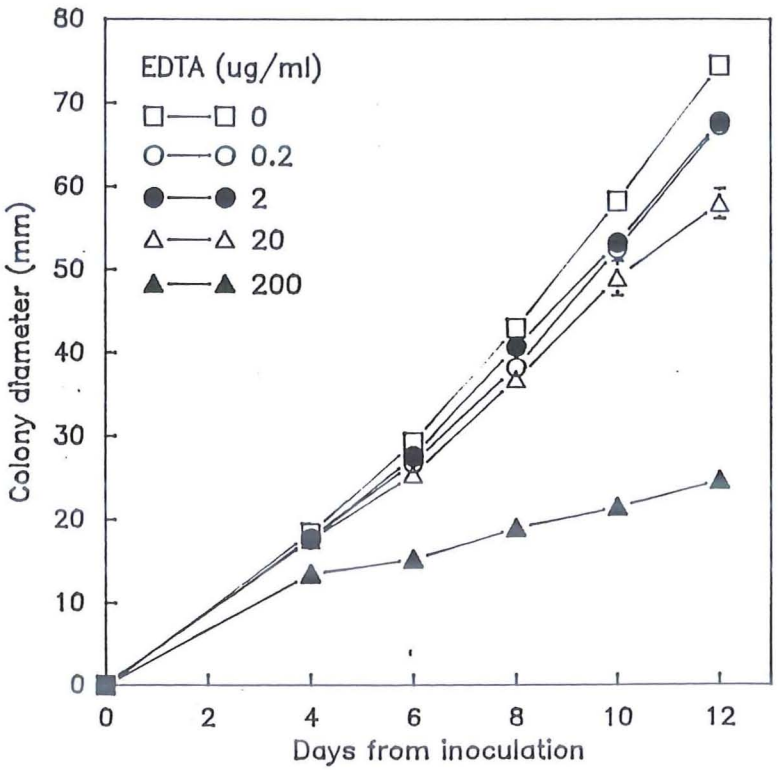


Fig. 4.19. The effect of EDTA on hyphal growth of *A. bisporus*. Data are means of four replicates and S.E. are shown when they exceed the diameter of the symbols.

Stimulation of mycelial growth by *P. putida* PMS118S and inhibition of growth by *P. tolaasii* PMS117S was apparent on all plates, at all concentrations of EDTA and EDDA, except 200 μg ml^{-1} EDTA,. At this concentration *P. tolaasii* PMS117S grew extremely poorly and did not inhibit mycelial growth. *P. putida* PMS118S grew well on CMM containing 200 μg ml^{-1} EDTA and hyphal growth was stimulated.

The effect of EDDA on mycelial growth of a range of fungi is shown in Table 4.4. None of the *Agaricus* spp., or strains, were able to grow on CMM containing EDDA, but weak hyphal extension did occur after a 10 μl drop of 0.1 M $FeCl_3$ was placed on the agar surface alongside the fungal inoculum. This occurred only on the plate containing 0.25 mg ml^{-1} EDDA. Interestingly, after the addition of $FeCl_3$ to the 0.25 mg ml^{-1} EDDA plate, *A. bisporus* (U3) markedly inhibited the growth of other fungi (*P. ostreatus, P. cystidiodes, A. campestris* and *Auricularia auricula*) growing on the same plate. Plating *P. putida* PMS118S alongside *A. bisporus* on CMM amended with EDDA did not reverse the conditions of iron starvation, despite the production of a fluorescent pigment (siderophore) by *P. putida*

Table 4.4. The effect of EDDA on mycelial growth of a range of basidiomycetes.

	Concentration of EDDA in CMM (mg ml^{-1})			
Fungal isolate	**0.25**	**0.5**	**1.0**	**2.0**
A. bisporus (U3)	-[a]	-	-	-
A. bisporus (649)	-	-	-	-
A. bitorquis (W2)	-	-	-	-
A. campestris	-	-	-	-
Pleurotus flabelatus	+++	++	+	+
P. eaus	++	++	+	+/-
P. ostreatus	+++	+/-	-	-
P. cystidiodes	+/-	-	-	-
Lentinus edodes	+++	+/-	-	-
Volvariella volvacea	+++	++	-	-
Coprinus bilanatus	+++	++	+	+
Flammulina velutipes	+++	+++	+++	+++
Auricularia auricula	++	+	+/-	-

Growth was assessed after 6 d incubation.

[a] +++, good growth; ++, moderate growth; +, weak growth; +/-, poor growth (just visible with the naked eye); -, no growth.

P. putida PMS118S grew at all EDDA concentrations and produced a pigment which fluoresced under UV light.

The effect of the P. putida *PMS118S siderophore on mycelial growth*

The freeze dried extract containing the pigmented siderophore produced by *P. putida* PMS118S inhibited mycelial growth of *A. bisporus*, and the control antibiotic assay discs containing freeze dried extract of succinate broth had no effect.

Iron status of CMM

A. flavescens (JG-9) grew on unamended CMM indicating the presence of hydroxamate type siderophores (Neilands 1984). The iron replete status of the medium was further indicated by the absence of a fluorescent pigment following growth of *P. putida* PMS118S.

The effect of non-living *P. putida* PMS118S cells on mycelial growth and basidiome initiation.

Incorporation of non-living P. putida *PMS118S cells into CMM*

The effect of non-living *P. putida* PMS118S on mycelial growth of *A. bisporus* is shown in Fig 4.20. In all instances CMM containing dead *P. putida* PMS118S cells inhibited the growth of *A. bisporus* mycelium.

Application on non-living P. putida *PMS118S cells to the surface of CMM*

P. putida PMS118S cells grown in CMM broth and KB broth, and killed either by heat, or chloroform treatment, inhibited both mycelial growth of *A. bisporus* on CMM, and mycelial growth and basidiome initiation of W19 (Fig. 4.21). No evidence of lysis of the bacterial colony by the advancing mycelium was observed.

The effect of culture filtrates on mycelial growth and basidiome initiation

Culture filtrates had no observable effect on the growth of *A. bisporus* mycelium or on basidiome initiation of the model strain, W19.

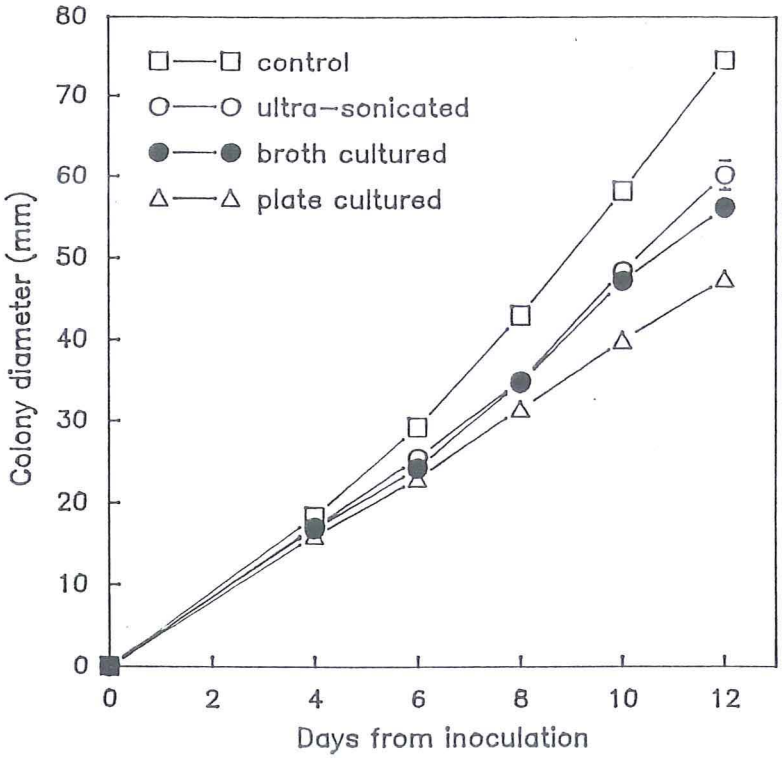


Fig. 4.20. The effect of non-living *P. putida* PMS118S (incorporated into CMM) on mycelial growth of *A. bisporus*. Data are means of four replicates and S.E. are shown when they exceed the diameter of the symbols.

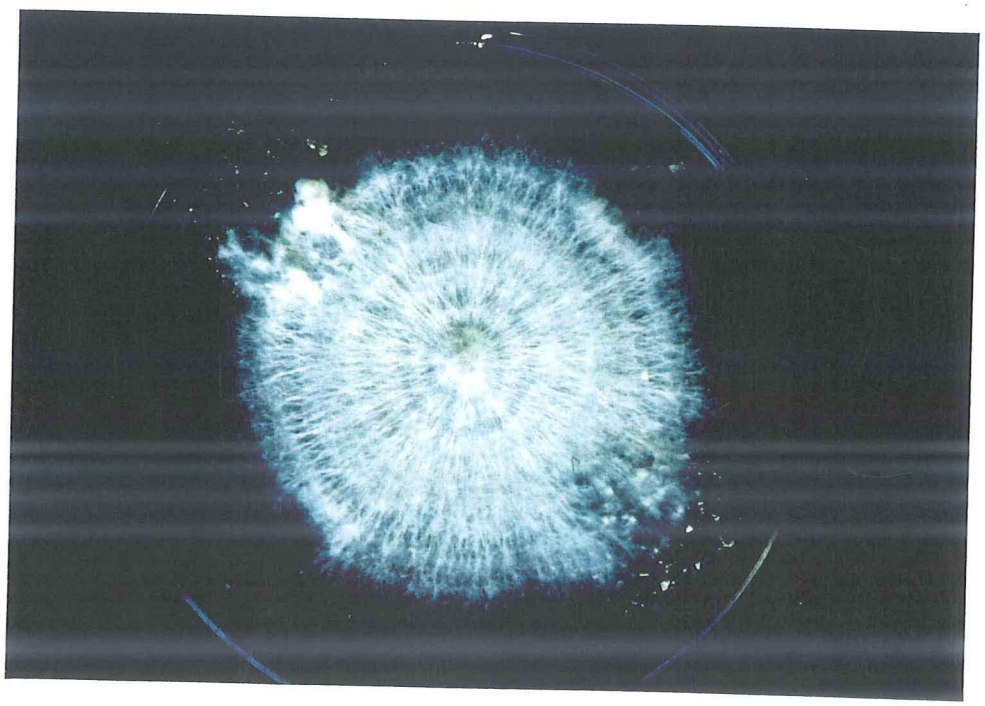


Fig. 4.21. The effect of non-living *P. putida* PMS118S on basidiome initiation of *A. bitorquis* W19. Chloroform killed KB cultured cells (bottom LH), chloroform killed CMM cultured cells (top RH), sterile peat supplemented with chloroform killed CMM cultured cells (top LH) and sterile peat supplemented with living *P. putida* PMS118S (bottom RH).

The transition from nutrient rich to nutrient poor media and its effect on mycelial growth and basidiome initiation.

The growth of *A. bisporus* mycelium from nutrient rich CMM to nutrient poor media is shown in Figs 4.22a and 4.22b. The growth of the fungal mycelium over dilute CMM was markedly slower than growth of the mycelium over water agar. Growth of the mycelium over full strength CMM was slightly slower than growth of the mycelium over water agar. The effect of *P. putida* PMS118S on hyphal growth is also shown in Figs 4.22a and 4.22b and on both water agar and dilute CMM stimulation of the rate of hyphal extension was apparent. The altered growth form stimulated by the bacterium is clearly discernible in the plates.

The growth form of *A. bisporus* hyphae on CMM in the presence of *P. putida* PMS118S and on CMM in the absence of *P. putida*, and on water agar, is shown in Fig. 4.23. The growth form of the hyphae in each instance is clearly different.

Growth of mycelium over a glass coverslip occurred, but the resulting growth was slow and confined to well developed mycelial strands which eventually spanned the coverslip. Growth reverted to normal once the barrier was traversed.

Hyphal growth experiments

The effect of a complete lawn of P. putida *PMS118S on hyphal growth of* A. bisporus

The rate of radial growth of *A. bisporus* mycelium was not promoted when *P. putida* PMS118S covered the entire surface of the medium. The rate of radial extension eventually increased, but not until after day six (Fig. 4.24)

Examination of the ability of hyphae growing in the presence of P. putida *PMS118S to retain the same rapidly extending growth form when sub-cultured and grown in the absence of the bacterium*

Rapidly extending mycelium, removed from above a *P. putida* PMS118S colony, did not continue to grow in this manner when cultured on a fresh plate in the absence of the bacterium.

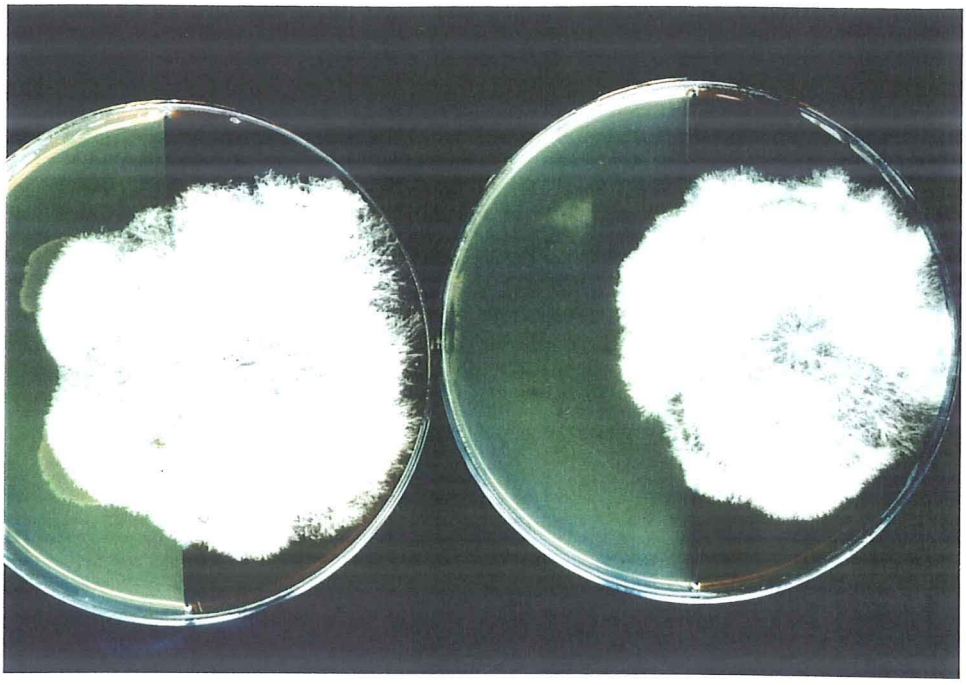


Fig. 4.22a The transition from CMM to dilute CMM and its effect on hyphal growth of *A. bisporus*. LH Petri dish contains *P. putida* PMS118S on the dilute medium and stimulation of mycelial growth by the bacterium is discernible.

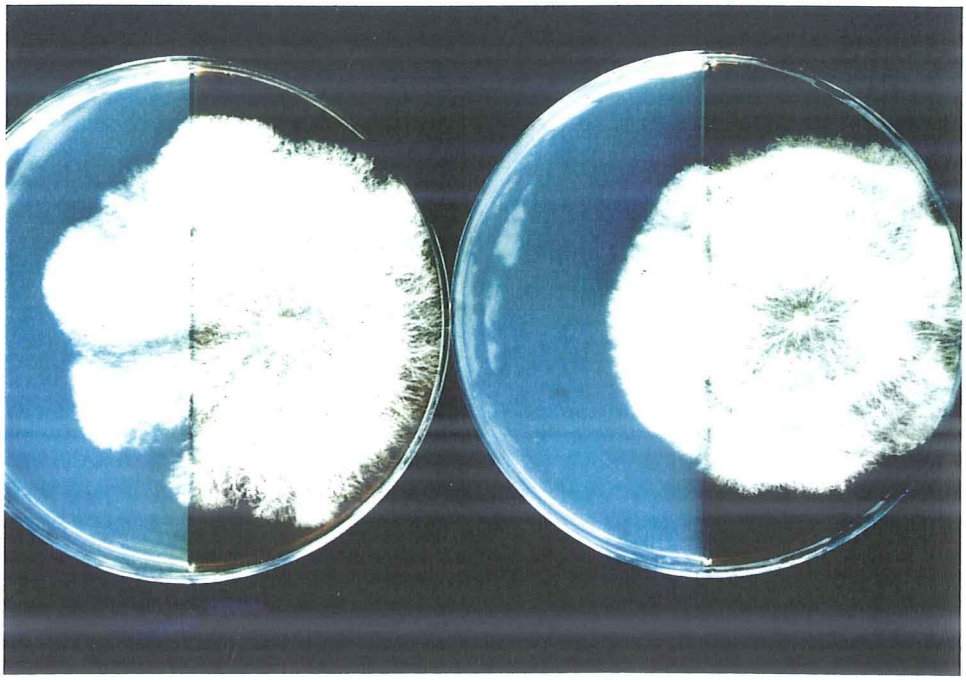


Fig. 4.22b. The transition from CMM to water agar and its effect on hyphal growth of *A. bisporus*. LH Petri dish contains *P. putida* PMS118S on the water agar and stimulation of mycelial growth by the bacterium is discernible.

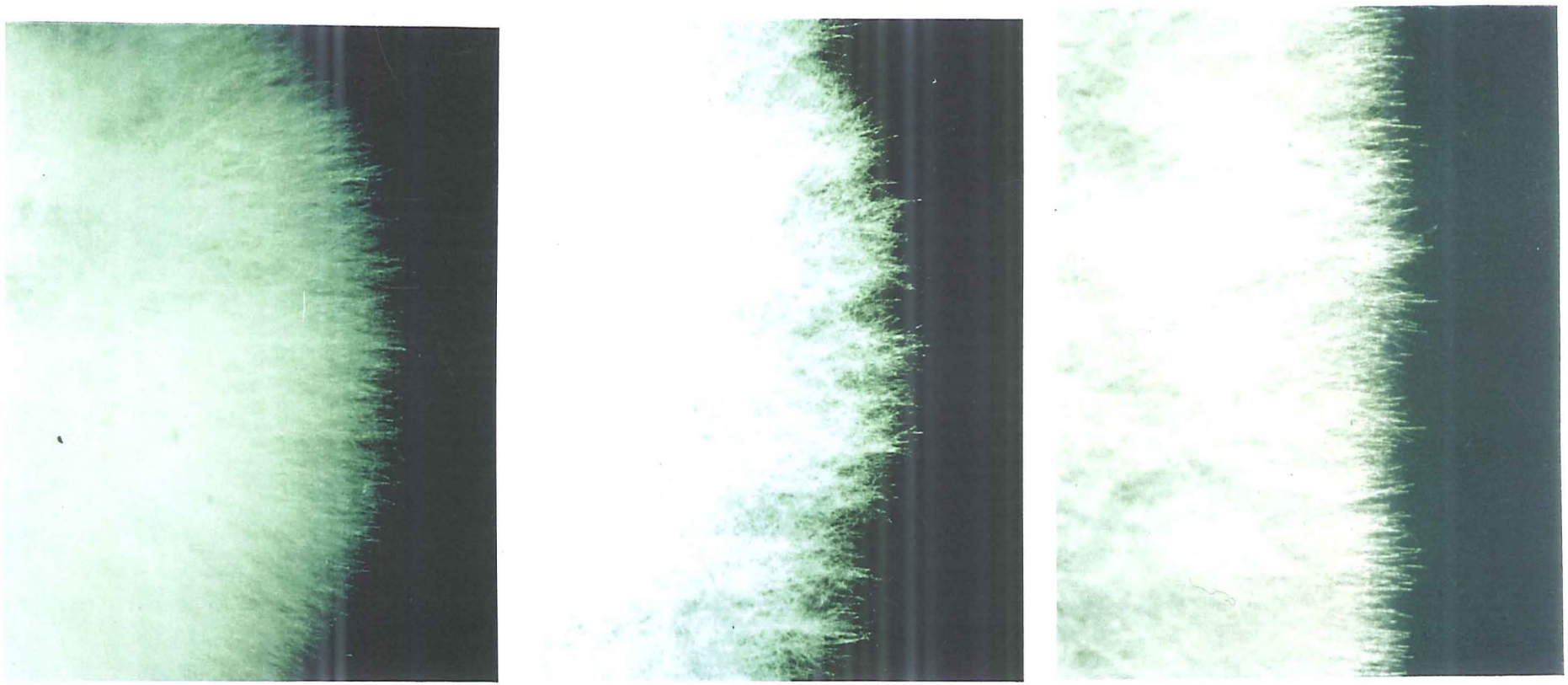


Fig. 4.23. The growth form of *A. bisporus* hyphae; (i) on CMM in the presence of *P. putida* PMS118S, (ii) on CMM in the absence of the bacterium, and (iii) on water agar in the absence of bacteria.

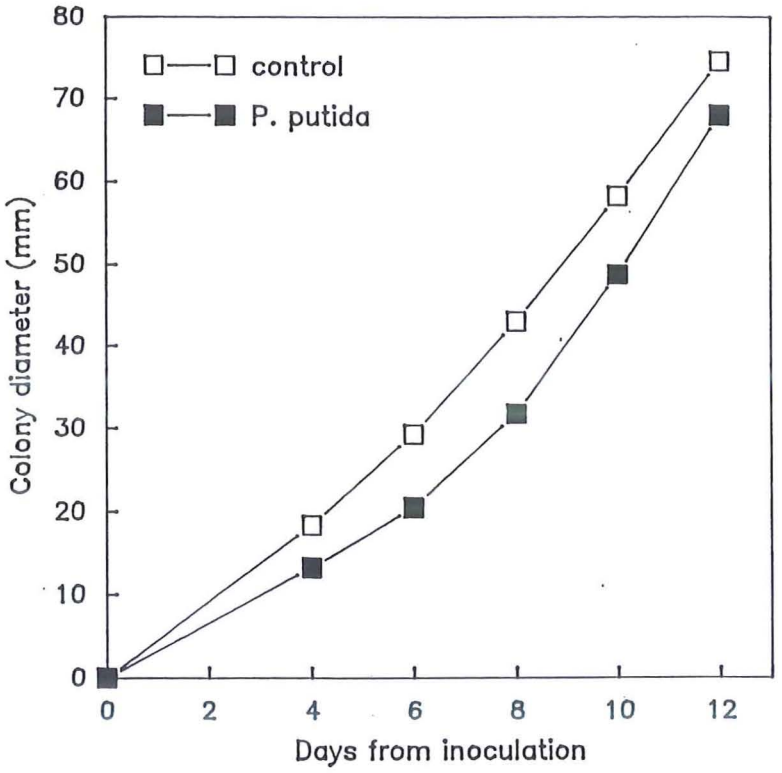


Fig. 4.24. The effect of a lawn of *P. putida* PMS118S on hyphal growth of *A. bisporus*. Data are means of four replicates and S.E. are contained within the symbols.

4.3.4. THE EFFECT OF *P. PUTIDA* PMS118S ON HYPHAL GROWTH OF *A. BISPORUS*

P. putida PMS118S markedly influenced the growth of *A. bisporus* mycellum. Figs 4.25 and 4.26 show the effect of the bacterium on the rate of expansion of *A. bisporus* colonies and Table 4.5 shows the effect of *P. putida* PMS118S on the morphology of leading hyphae at the margin of the fungal colonies. The increase in the rate of radial growth was accompanied by an increase in the SIL, but hyphal diameter was not affected. Fig. 4.27 shows the relationship between mycelial branching, as indicated by the SIL, and growth rate. The correlation coefficient ($r = + 0.98$) between these two variables was highly significant ($P < 0.001$).

The branch angle of hyphae growing in the presence of *P. putida* PMS118S varied between 10° and 45° and hyphae were collaterally aligned, regardless of the initial branch angle. The angle subtended by leading and primary hyphae growing in the absence of *P. putida* PMS118S, and on control plates, was between 30° and 85° and resulting hyphal growth was divergent. The balance between aerial and submerged hyphae was also affected by *P. putida* pms118S and changed from predominantly submerged to largely aerial. This was noticeable with the naked eye.

Table 4.5. The effect of *P. putida* PMS118S on the expansion of *A. bisporus* colonies.

Treatment	Colony radial[a] growth rate ($\mu m\ h^{-1}$)	Subapical[b] internode length (μm)	Hyphal[c] diameter (μm)
Presence of *P. putida*	170*	470 ± 15*	5.4 ± 0.05
Absence of *P. putida*	90	245 ± 9	5.5 ± 0.05
Control	100	253 ± 9	5.5 ± 0.05

Note SIL and hyphal diameter were measured 9 d after inoculation with *A. bisporus* (3 d after inoculation with *P. putida*).

[a]Mean of 10 colonies.

[b]Mean and standard error of 5 replicate plates.

[c]Mean and standard error of 10 leading hyphae of 5 replicate plates.

*Significantly different ($P < 0.001$).

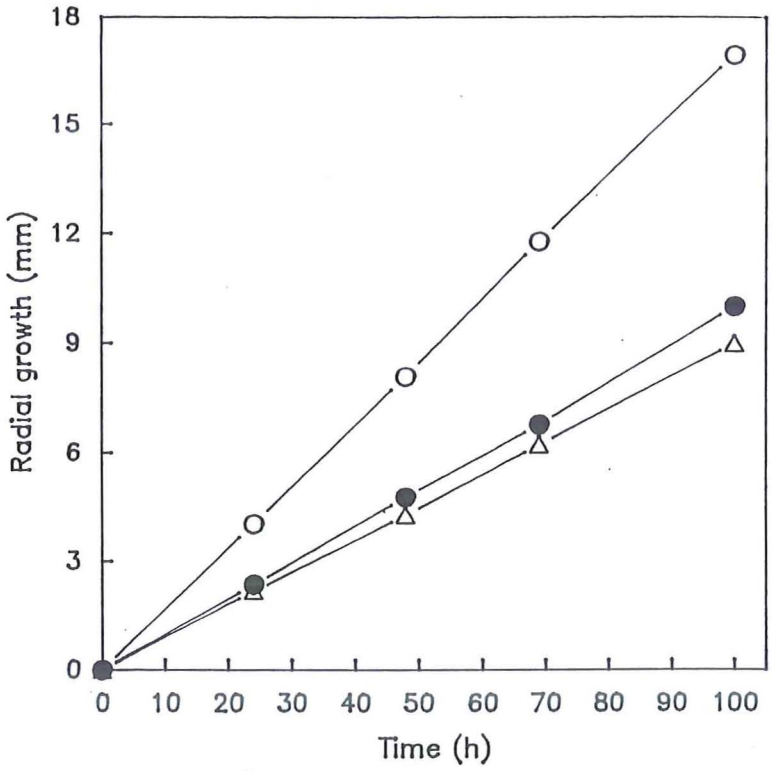


Fig. 4.25. The effect of *P. putida* PMS118S on the rate of extension of *A. bisporus* hyphae. Growth of hyphae in the presence of *P. putida* (○), in the absence of *P. putida* (△), and on control plates (●). Measurements were made after the fungus had been growing in the presence of the bacterium for 60 h. Data are means of 10 replicates and S.E. are contained within the symbols.

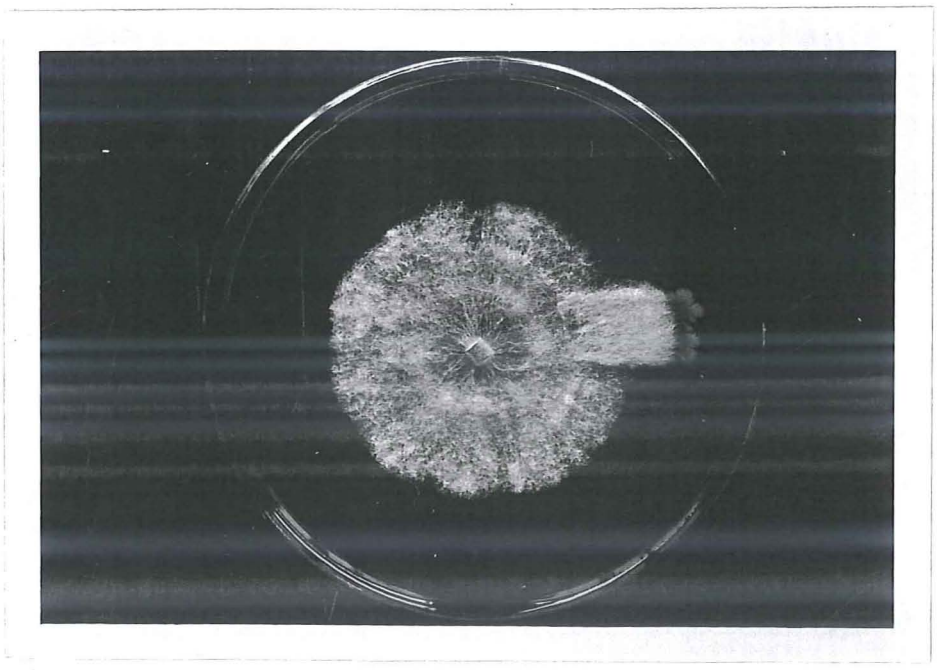


Fig. 4.26. The effect of *P. putida* PMS118S on the rate of extension of *A. bisporus* mycelium.

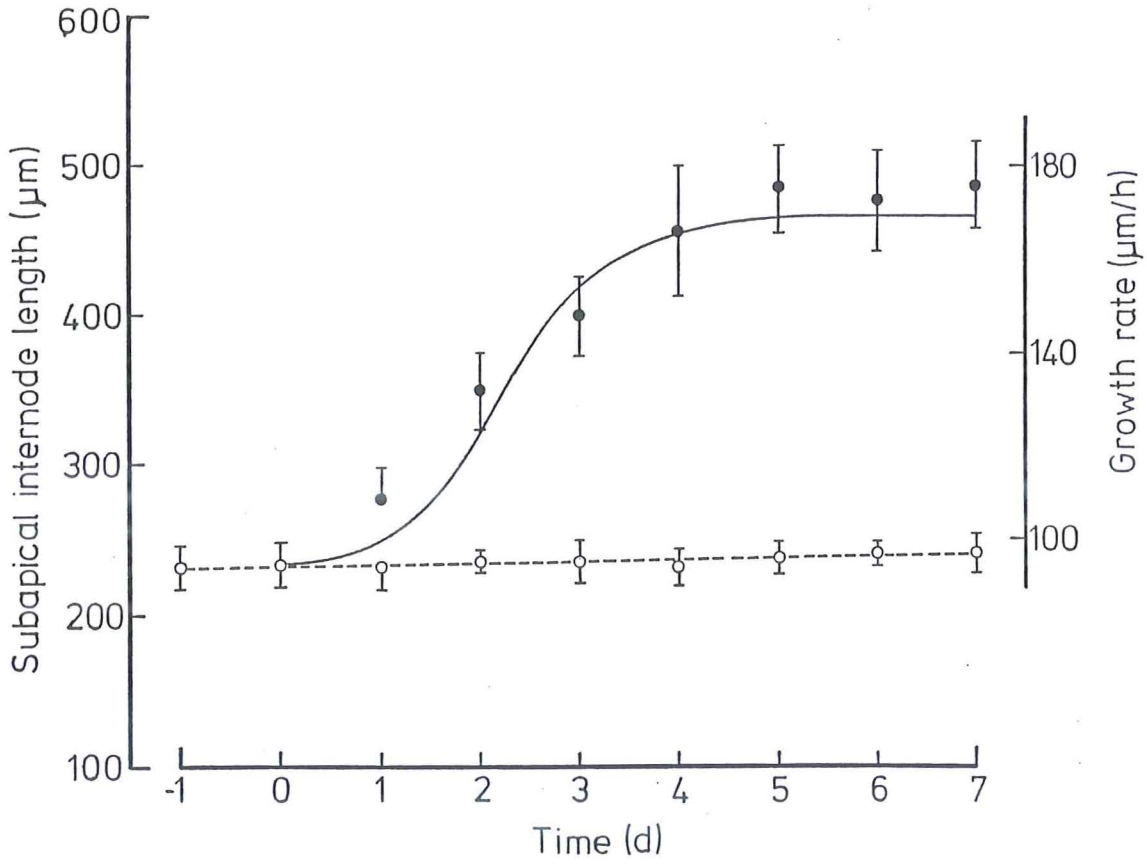


Fig. 4.27. The effect of *P. putida* PMS118S on subapical internode length (SIL) and on the rate of extension of *A. bisporus* hyphae. Data are means of 10 replicates (± S.E.) taken from a single Petri dish. ANOVA revealed a significant difference between treatments ($P < 0.001$) and the effect of time was significant ($P < 0.01$); the interaction effect was not significant. SIL for hyphae growing in the presence of *P. putida* (●), SIL for hyphae growing in the absence of *P. putida* (O). Growth rate of hyphae growing in the presence of *P. putida* (——), growth rate of hyphae growing in the absence of *P. putida* (----).

Mycelial strands became visible several days after colonization of a bacterial colony was complete. These structures were usually more pronounced and numerous in the vicinity of the bacterial colonies.

The effect of *P. putida* PMS118S on mycelial dry weight per unit area of substrate is shown in Table 4.6 and Fig. 4.28 shows the Petri dishes immediately prior to sampling. The rate of radial growth of *A. bisporus* mycelium and the dry weight of fungal tissue produced per unit area of substrate was used to calculate an estimate of the dry weight of fungal material produced per time interval. This information is presented in Table 4.7. *P. putida* PMS118S caused the dry weight of mycelium produced per unit area of substrate to decrease, but did not effect the dry weight of fungal tissue produced per unit time. The dry weight of mycelium produced by the fungus growing in the absence of *P. putida* PMS118S was affected by the area colonized by the bacterium. When *P. putida* PMS118S occupied half of the uncolonized area of the plate (Fig. 4.3), the dry weight of fungal material produced per unit area of substrate and per unit time, was significantly less than that produced on control plates.

Table 4.6. The effect of *P. putida* PMS118S on dry weight of *A. bisporus* mycelium

Treatment	Dry weight of mycelium in , four 10 mm diam, agar-mycelial plugs (mg)
Presence of *P. putida* (100 % of available area)	1.60 + 0.08
Presence of *P. putida* (50 % of available area)	1.78 + 0.04
Absence of *P. putida* (50 % of available area))	1.72 + 0.07
Presence of *P. putida* (25 % of available area)	1.82 + 0.06
Absence of *P. putida* (75 % of available area)	3.55 + 0.08*
Control	3.60 + 0.09*

Measurements were made 10 d after inoculation with *A. bisporus* (4 d after inoculation with *P. putida*)
Data are means and standard errors of eight replicates.
*Significantly different ($P < 0.001$).

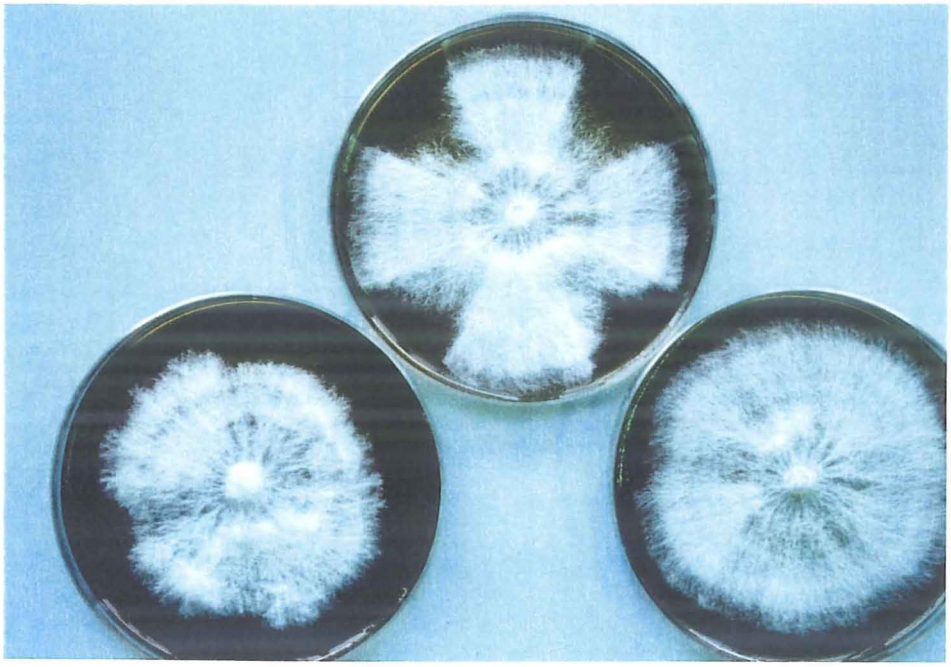


Fig. 4.28. The effect of *P. putida* PMS118S on hyphal growth of *A. bisporus* prior to sampling and determining dry weight of fungal tissue. Top plate shows *P. putida* covering 50 % of the available area, bottom LH is a control and bottom RH shows *P. putida* covering 100 % of the available area (see section 4.2.5.).

Table 4.7. The effect of *P. putida* PMS118S on the dry weight of *A. bisporus* mycelium, and area colonized by the mycelium, over a four day period.

Treatment	Colony radial growth rate ($\mu m\ h^{-1}$)	Area covered by mycelium (mm^2)	Area covered by mycelium per quarter plate (mm^2)	Dry weight of mycelium produced per treatment (mg)	Dry weight of mycelium produced per quarter plate (mg)
Plate i					
Presence of *P. putida* (100 %)[b]	175	2470	618	12.6[a]	3.2
Plate ii					
Presence of *P. putida* (50 %)[b]	170	1195	598	6.8	3.4
Absence of *P. putida*	88	507	254	2.8 9.6[a]	2.8
Plate iii					
Presence of *P. putida* (25 %)[b]	170	598	598	3.5	3.5
Absence of *P. putida*	93	818	273	9.2 12.7[a]	3.1
Control	100	1194	398	13.7[a]	3.4

Measurements are estimates calculated from the growth rate and dry weight of mycelium per unit area. A colony diameter of 30 mm (6 d growth) was assumed at the start of the 4 d period.

Dry weight per quarter plate and area covered per quarter plate are given for comparative purposes

[a]The total dry weight of mycelium produced per plate in 4 d.

[b]Refers to the area of the Petri dish colonized by *P. putida* (see text, section 4.2.5.)

4.4. DISCUSSION

4.4.1. COMPOST MALT MEDIUM

The results presented in section 4.3.1. demonstrate the suitability of CMM as a laboratory medium for the growth of *A. bisporus*. The rapid and vigorous mycelial development promoted by CMM is most likely attributable to the medium's resemblance to the commercial substrate of the fungus. This property renders CMM invaluable for *in vitro* studies concerned with the biology of *A. bisporus*.

Radial growth of fungal colonies occurs at a constant linear rate (Cochrane 1958), provided there is an excess of all nutrients within the growth substrate and an absence of inhibitory substances (Trinci 1984). The results indicate that substrates commonly used for the culture of *A. bisporus* are either, unable to provide the fungus with the nutrients required to support a constant growth rate, or promote the build up of fungal metabolites which are inhibitory to the growth of the mycelium. The use of these media for routine culture of *A. bisporus* should be re-examined.

4.4.2. *IN VITRO* FRUITING OF *A. BISPORUS*: A MODEL SYSTEM

The results presented in Table 4.1 indicate the suitability of W19 to function as a model strain for studies concerned with the involvement of bacteria in basidiome initiation of *A. bisporus*. While it is not possible to correlate fruiting of *A. bisporus* in commercial cultivation with fruiting of W19 on CMM, the identical response of the two agarics to the *Pseudomonas* isolates, suggests that the mechanism controlling fruiting of W19 is similar to that of *A. bisporus*. The ability of W19 to form primordia on CMM in Petri dish culture, when grown in association with basidiome stimulatory bacterial isolates, is invaluable for studies on the role of bacteria in the process of basidiome initiation of commercial *A. bisporus* strains. Its simplicity permits the screening of large numbers of bacteria which enables genetical and molecular studies to proceed.

A small number of primordia were produced in the modified Halbschalentest in the absence of basidiome promoting bacteria. The production of primordia by axenic cultures of

A. bisporus was previously described by Wood (1976) and demonstrates that the requirement of bacteria for fruiting is not absolute.

Eger (1972) and Rainey & Cole (1987) reported stimulation of the rate of linear extension of *A. bisporus* hyphae by *P. putida* prior to the appearance of primordia in Halbschalentests. Stimulation of the rate of mycelial growth by *P. putida* on CMM appears to reflect this event. The results presented in Table 4.1 reveal a close association between the ability of bacteria to stimulate mycelial growth and their ability to promote basidiome initiation. It is possible that the stimulation of mycelial growth represents an initial stage in the process of basidiome differentiation (see section 4.4.4.). The inability of *A. bisporus* to enter the primordia initiation phase of the fruiting process when cultured on CMM provides an opportunity to examine in detail the effect of *P. putida* on hyphal growth of the fungus during the preliminary phase of the interaction.

The failure of *A. bisporus* to initiate primordia on CMM when cultured in association with basidiome promoting bacteria is not surprising. It is possible that the unavoidably high carbon dioxide concentrations within the Petri dish are largely responsible for the inability of the fungus to fruit under these circumstances (Long & Jacobs 1969). In commercial cultivation, fruiting of W19 is not inhibited by high carbon dioxide concentrations (personal communication, J. F. Smith, I.H.R.) and this may partly explain the unique ability of this strain to fruit in Petri dish culture.

The preliminary investigation aimed at determining the range of bacteria able to promote basidiome initiation of *A. bisporus* was undertaken to further demonstrate the usefulness of the model system. Although only a small number of isolates were examined, the ability to promote basidiome initiation was confined to certain pseudomonads isolated from the casing layer. Known mushroom pathogens, such as *P. tolaasii* and *P. agarici*, failed to promote fruiting, as did a range of other soil inhabiting bacteria. Valuable information would be gained by including this assay in future studies aimed at examining the taxonomy of casing layer inhabiting bacteria.

It was interesting to observe lysis of bacterial colonies by mycelium of *A. bisporus* and W19. This may have been due to the production of extracellular degradative enzymes, but this

remains to be determined. *A. bisporus* is able to utilize dead bacteria as a source of nutrients (Fermor & Wood 1981, Grant *et al.* 1984) and these observations indicate that the fungus also possesses the ability to kill bacteria. The ability to kill competing micro-organisms may provide *A. bisporus* with an ecological advantage and helps to explain how this relatively slow growing fungus is able to dominate in a non-sterile and competitive environment. It would be interesting to examine this aspect further and to investigate the interactions between *A. bisporus* and other soil/compost inhabiting fungi.

The lysis of fluorescent *Pseudomonas* colonies was rare and may be related to their protective lipopolysaccharide layer which accounts for their marked resistance to a range antimicrobial compounds and detergents (Nikaido & Hancock 1986). The ability of basidiome stimulatory pseudomonads to resist the harmful compounds produced by the fungus provides further evidence for a close association between the two organisms and also helps to explain the dominance of pseudomonads within the casing layer environment (Cresswell & Hayes 1979).

4.4.3. INVESTIGATIONS INTO THE NATURE OF THE MICROBIAL STIMULUS EFFECTING BASIDIOME INITIATION OF *A. BISPORUS*

The effect of activated charcoal on hyphal growth and basidiome initiation

The results presented in Table 4.3 provide further confirmation of the work of Eger (1961) and others who have demonstrated the ability of activated charcoal to replace the effects of bacteria under axenic conditions. Activated charcoal was not as effective at promoting fruiting as a viable microflora and fewer primordia were produced which took longer to initiate.

The ability of activated charcoal to promote primordia production by W19 on CMM further indicates the suitability of W19 to function as a model strain. The improved fruiting response when either activated charcoal or *P. putida* was incorporated into sterile peat is presumably related to the more *in vivo* like conditions afforded by the peat.

Much has been assumed by workers who have drawn conclusions concerning the mechanism by which bacteria promote fruiting, from the ability of activated charcoal to trigger the same response. While activated charcoal is able to readily absorb low molecular weight compounds, it may also release compounds and provides surfaces on which reactions can take

place. It is possible that activated charcoal may trigger fruiting by a mechanism which is entirely different from the mechanism by which *P. putida* promotes basidiome initiation and may be unrelated to the ability of activated charcoal to function as a general absorbent. Caution needs to be exercised in interpreting the results of activated charcoal experiments, nevertheless, as suggested by Wood (1976, 1982), the ability of activated charcoal to replace the effects of *P. putida* indicates that the stimulus afforded by the bacterium is of a negative nature.

Subjecting *A. bisporus* mycelium to activated charcoal before providing it with an opportunity to exploit the available nutrient source, resulted in inhibition of mycelial growth, however, when the fungus encountered the reproductive stimulant after 5 d growth, the rate of linear extension of hyphae was increased. These results provide some indication of the complex interrelationship which has been shown to exist between mycelial domain, resource pool and reproductive commitment (Rayner *et al*. 1984). Reproduction can not take place until a resource unit of sufficient size to support the production of basidiomata has been exploited. Incorporating activated charcoal into the growth substrate may have caused the fungus to attempt to enter the reproductive phase of growth without having first exploited the available resource unit. Consequently, growth was inhibited and as the amount of activated charcoal within the medium was increased (and therefore the strength of the reproductive signal), the growth of *A. bisporus* was correspondingly decreased. A similar result was found when *A. bisporus* was subjected to the reproductive signal from *P. putida* (section 4.3.3.) before being given an opportunity to exploit the nutrients available within the substrate. When the reproductive signal was provided after resource exploitation had occurred, the response of the fungus was typical of normal reproductive growth (see also section 4.4.4.).

The *in vitro* fruiting systems based on CMM provide an opportunity to examine more closely the relationship between the size (quality) of the resource unit exploited and the ability of the fungus to enter the reproductive phase of growth.

The effect of iron and iron chelating agents on mycelial growth and basidiome initiation

The failure of $FeCl_3$, EDTA, EDDA and the *P. putida* siderophore to promote basidiome initiation provides further confirmation of the work of Wood (1976) and conflicts with the findings of Hayes (1972, 1981).

The conclusions drawn by Hayes (1972, 1974, 1981) concerning the involvement of iron in basidiome initiation are difficult to understand, particularly when considered in the light of current knowledge concerning iron and siderophores. Many of the experiments conducted by Hayes were performed under imprecise conditions and without proper understanding of the complex reactions and interactions involved. The emphasis Hayes placed on ferrous iron provides an example of this: Under aerobic, oxidising conditions, such as exist in plate culture, ferrous iron is rapidly oxidized to the ferric form and precipitated as oxyhydroxides (Neilands 1984), or bound to naturally occurring chelating agents, such as amino acids, organic acids and phosphates. It is this bound and/or chelated ferric iron with which micro-organisms living in the majority of environments must contend and it is the ferric form toward which siderophores display considerable affinity (Neilands 1984). In contrast to the ideas of Hayes, the ferrous form has little relevance in alkaline, aerobic environments and addition of ferrous salts to culture media does not approximate the effect of iron solubilization by *P. putida* siderophores.

The involvement of iron in basidiome initiation, as suggested by Hayes, pre-supposes an iron deplete environment (this is necessary to stimulate siderophore production by micro-organisms (Neilands 1984)). Peats, however, contain large amounts of iron, a consequence of their high organic content (Duchaufour 1982). Ganney & Richardson (1974) reported substantial amounts of iron in peat casing layers and Hayes (1981) found a significant level of water soluble (readily available) iron in a 'paper mill by-product' (PMB) casing soil. It is quite possible that the casing layer contains sufficient available ferric iron to suppress microbial siderophore production. If this is shown to be true, then the involvement of iron in mushroom fruiting would appear even less likely.

The ability of *A. bisporus* to grow in the presence of EDTA suggests that it produces a siderophore which has an affinity for ferric iron which is greater than EDTA. Its inability to grow in

the presence of EDDA, indicates that the siderophore has a stability constant of less than $\log_{10}K$ 33.9. The siderophore produced by *P. putida* PMS118S was able to sequester iron from EDDA and therefore must have a stability constant in excess of $\log_{10}K$ 33.9. On the basis of these results it is possible to construct a tentative hierarchy of ferric iron binding compounds: *P. putida* PMS118S siderophore > EDDA > *A. bisporus* siderophore > EDTA. A similar hierarchy was reported by Scher (1986): *Pseudomonas* siderophores > EDDA > *Fusarium* siderophores > EDTA. The binding affinities predict that ferric iron held by EDDA or the *P. putida* PMS118S siderophore would not be available to *A. bisporus*. The inhibition of mycelial growth by the *P. putida* PMS118S siderophore is consistent with this prediction. Also consistent with this, was the inability of *A. bisporus* to utilize the siderophore produced by *P. putida* PMS118S to reverse conditions of iron starvation on CMM amended with EDDA. The inability of *P. putida* PMS118S to inhibit mycelial growth on CMM or in peat (Rainey & Cole 1987) further suggests, as discussed above, that the casing layer is not iron limited.

It would be interesting to investigate siderophore production by *A. bisporus* and to determine the ability of *P. putida* PMS118S to utilize the fungal siderophore. It would also be worthwhile to examine the casing layer for the presence of *Pseudomonas* siderophores.

The effect of non-living *P. putida* PMS118S cells on mycelial growth and basidiome initiation

The results from this study demonstrate that basidiome initiation is triggered only by living *P. putida* PMS118S cells. This further indicates that the interaction between the two organisms is of a specific nature. It also demonstrates that some function, confined only to living cells and most likely of a metabolic nature, provides the stimulus which triggers reproductive development.

It was interesting to note the marked inhibitory effect of dead cells on the growth of the mycelium, especially the increased inhibitory effect of cells grown in plate culture. Leakage of toxic metabolites by the dead bacteria provides the most plausible explanation for the inhibition, but if this is true, then greatest inhibition would be expected by the cells which were disrupted by ultra-sonication. This result highlights the effect of culture conditions on the properties of micro-organisms.

The inhibition of mycelial growth by non-living *P. putida* PMS118S provides an explanation for the inhibition of mycelial growth by this organism on 2 % malt extract (Bacto) reported by Rainey & Cole (1987). Bacto malt extract supports poor growth of *P. putida* (and *A. bisporus*, see section 4.3.1.) and after several days growth the bacterial colony rapidly discolours due to the death of a large number of cells. In the light of the above findings, these dead cells were probably responsible for the observed inhibition.

The requirement of *A. bisporus* for living *P. putida* indicates the need for the mushroom to provide the bacterium with conditions which favour its survival.

The effect of culture filtrates on mycelial growth and basidiome initiation

Culture filtrates had no observable effect on either mycelial growth or basidiome initiation. Wood (1976), in contrast to Park & Agnihotri (1969a) and Hayes (1972), was also unable to detect any effect of culture filtrates on mushroom fruiting. These experiments do not exclude the possibility that *P. putida* PMS118S produces a volatile fruit body-promoting compound (this would be lost during the freeze-drying process). The involvement of such a compound however, appears unlikely. Peerally (1979) and Rainey & Cole (1987) showed that fruiting of *A. bisporus* in response to *P. putida* was confined to the region where the two organisms co-existed. This suggested that if the bacterium does produce a 'fruit body-inducing' compound, then it is of low volatility. The results presented in section 4.3.2. also indicate this. Furthermore, if a 'fruit body-inducing' compound was produced then a gradient would be created within the agar and the fruiting response would most likely be detectable at a distance from the bacterial colony. This was never observed and the reproductive response was always confined to the region where the bacteria were growing. In addition, the production of a fruit body inducing compound by *P. putida* is not consistent with the observed effects of activated charcoal on basidiome initiation.

The transition from nutrient rich to nutrient poor media and its effect on mycelial growth and basidiome initiation

Growth from a nutrient rich environment to several different nutrient poor environments was demonstrated and is consistent with the known ability of *A. bisporus* mycelium to translocate nutrients over considerable distances (Nielsen & Rasmussen 1962, Flegg 1981). The rate of

mycelial extension was not stimulated by the nutrient poor zone, as might have been expected if the difference between the nutrient rich and poor environments triggered the onset of reproductive growth, as suggested by Couvy (1974).

An increase in the rate of mycelial extension can occur in response to nutrient limitation (Thompson & Rayner 1983) and is thought to represent an attempt by fungi to locate new nutrient pools (Cooke & Rayner 1984). It was possible that the response of *A. bisporus* hyphae *to P. putida* PMS118S, observed during the initial stages of the interaction, was typical of this behaviour, but these results indicate that this is unlikely.

Stimulation of the rate of hyphal extension by *P. putida* PMS118S on dilute CMM was expected, as this substrate supports growth of the bacterium, and live (metabolically active) bacteria appear necessary to trigger this response (section 4.3.3.). On water agar there was no visible evidence of bacterial growth, yet mycelial growth was also stimulated by the bacterium. Examination of the water agar section of the Petri dish, containing both mycelium and *P. putida* PMS118S, revealed obvious signs of bacterial growth.

Fungi are known to leak nutrients from their hyphae (Lockwood & Filonow 1981) which can be metabolized by bacteria and *A. bisporus* mycelium has been shown to produce volatile compounds which can be utilized by bacteria for growth (Hayes *et al.* 1969). This observation demonstrates that under conditions of nutrient limitation, such as exist in the casing layer, *P. putida* is able to derive considerable benefit from *A. bisporus*. In return for providing *P. putida* with nutrients for growth, *A. bisporus* receives the stimulus initiating the onset of reproductive development. This observation provides evidence of a balance of benefit in the relationship between the two organisms which helps to explain the evolution of this unique relationship.

Comments on nutrient stress as a trigger for reproductive growth.

Ingratta & Patrick (1987) suggested that basidiome initiation occurred in response to microbially induced nutrient stress and ensuing fungistatic effects. Couvy (1974), Lambert (1938) and Long & Jacobs (1974) also suggested that fruiting was a response to nutrient stress. If fruiting does occur in response to nutrient stress then it should be possible for basidiome initiation to occur in the absence of bacteria, but this is rarely seen. Mycelium growing on compost under

commercial cultivation must eventually become nutrient stressed, but without the casing layer and associated microflora few fruit bodies are ever produced. Similarly, axenic Petri dish cultures of *A. bisporus* would be expected to produce primordia, but this is infrequently observed (Wood 1976) (and has never been seen in this laboratory, even after prolonged incubation (12 wk)). Furthermore, if microbially induced stress provides the trigger for basidiome initiation, then bacteria which inhibit the growth of *A. bisporus* on CMM would be expected to promote fruiting, but this is not found (see section 4.3.2.) These observations and the results discussed above, indicate that fruit body initiation is unlikely to occur in response to microbially induced nutrient stress.

Examination of the ability of hyphae growing in the presence of *P. putida* PMS118S to retain the same rapidly extending growth form when sub-cultured and grown in the absence of the bacterium

In fungi, such as *Serpula lacrimans* (Coggins *et al.* 1980) and *Ceratobasidium cereale* (Kataria 1988), which display a transition between slow-dense and fast-effuse growth forms, the fast 'sectored' growth form is usually stably maintained after sub-culturing. Failure of the sub-cultured mycelium to revert to its previous form suggests that differentiation has occurred (Rayner & Coates 1987). Differentiation of this type was not apparent in the rapidly extending hyphae of *A. bisporus* as the mycelium reverted to its previous form when sub-cultured in the absence of *P. putida* PMS118S.

4.4.4. THE EFFECT OF *P. PUTIDA* PMS118S ON HYPHAL GROWTH OF *A. BISPORUS*: A PRELIMINARY STAGE IN THE PROCESS OF BASIDIOME MORPHOGENESIS

The results from this set of experiments revealed the marked affect of *P. putida* PMS118S on hyphal growth and colony morphology of *A. bisporus*. *P. putida* PMS118S promoted the rate of radial hyphal extension, suppressed the frequency of branching, caused the branch angle, alignment of hyphae and balance between aerial and submerged hyphae to change, but did not affect hyphal diameter. These effects were not apparent in mycelium which did not grow in direct contact with *P. putida* PMS118S. Mycelium growing on the same plate as the bacterium, but not in direct contact with *P. putida* PMS118S, displayed a reduction in the rate of radial growth and in the frequency of branching when compared to the growth of *A. bisporus* on control plates.

The SIL proved to be a consistent measure of mycelial branching in *A. bisporus* and provided a means of describing branching in colonies where the density of growth made it impossible to measure more than a single apical internode, or intercalary compartment, from a leading hyphae. A positive correlation between branching and colony radial growth rate, has also been reported for a temperature sensitive mutant of *Neurospora crassa* (Steele & Trinci 1977) and *Rhizoctonia cerealis* (Trinci 1985).

Interpretation of the response of hyphae to P. putida *PMS118S*

The growth of *A. bisporus* hyphae in the presence of *P. putida* PMS118S was characteristic of the fast-effuse growth form exhibited by some fungi. This mycelial mode is often associated with domain extension and exploration and contrasts with the slow-dense mycelial growth form, more often associated with resource exploitation and consolidation of territorial gains (Gregory 1984, Rayner *et al.* 1985a, Rayner & Coates 1987). The change to a rapidly extending growth form was accompanied by a switch to coherent collateral growth which is indicative of the preliminary stage in the development of linear vegetative organs, such as mycelial cords and strands (Rayner *et al.* 1985b).

Mycelial strands are frequently associated with growth of fungi over nutrient poor zones (Butler 1958, Mathew 1961, Watkinson 1979, Rayner *et al.* 1985b) and provide channels for translocation of water and nutrients (see Jennings (1984) and Thompson (1984)). They are also thought to function as nutrient reservoirs (Hein 1930, Sarazin 1955, Watkinson 1984). While mycelial strands are typically associated with vegetative growth, they are also found in connection with fruit bodies where they serve to link basidiomata with a food base (Thompson 1984, Rayner *et al.* 1985a). The association between fruit bodies and mycelial strands is particularly apparent in the commercial mushroom casing layer and primordia formed on CMM by *A. bitorquis* (W19) were also linked to the mycelial mat via mycelial strands (Fig. 4.6).

The formation of mycelial strands in the vicinity of the bacterial colonies also indicates that *P. putida* PMS118S is not a utilizable nutrient source. If *P. putida* PMS118S was providing the fungus with nutrients, then a change in mycelial growth consistent with a nutrient rich zone would

be expected, that is, a decrease in the rate of hyphal extension and an increase in the frequency of branching (Flegg & Wood 1985).

The response of *A. bisporus* hyphae to *P. putida* PMS118S can be interpreted in terms of reproductive growth as follows:- Upon receipt of the reproductive stimulus from *P. putida*, *A. bisporus* begins to prepare the foundation for basidiome initiation. This involves rapid extension of collaterally aligned hyphae which results in colonization of the area over which primordia can initiate and leads to the formation of mycelial strands. The mycelial strands provide channels in which nutrients can be stored and through which substrates necessary for basidiome development can be quickly transported, once initiation of primordia occurs.

Colonization of the area over which reproduction can occur

Successful reproduction is dependent upon many factors, including the size and quality of the resource unit exploited, the availability of nutrients and a range of environmental parameters. An additional factor which may provide an indication of reproductive fitness, is the area colonized by the fungus, over which reproduction can occur. Maximizing this area will ensure, that for any given resource unit, the maximum number of fruit bodies will be produced. This is an important consideration for all fungi, especially for those which exist in environments where long term vegetative survival cannot be guarantied and where the best reproductive strategy is to produce as many fruit bodies as possible, in a single flush, as soon as conditions are favourable.

A. bisporus appears to have responded to *P. putida* PMS118S in a manner which maximizes the area over which fruit body initiation can occur. The rapid hyphal extension triggered by *P. putida* PMS118S led the fungus to colonize a considerably greater area per unit time, than it colonized on either control plates, or in the absence of *P. putida* PMS118S (Table 4.7). This rapidly extending mycelium was always confined to the area above the bacterial colony and the rate of colonization remained relatively constant, regardless of the area occupied by the bacterium (Table 4.7). When growing in the absence of the bacterium, the fungus did not to behave in this manner, yet it possessed the potential and resources to do so. The marked preference that *A. bisporus* exhibited for growing in the presence of *P. putida* PMS118S suggests that it must be benefiting from the association.

Interpretation of the results from the dry weight of fungal tissue determinations

The calculated dry weight of *A. bisporus* mycelium produced after four days growth in the presence of *P. putida* PMS118S was very similar to that produced over a comparable sector, on control plates. The dry weight of mycelium produced in the presence of *P. putida* PMS118S was little affected by the area occupied by the bacterium, but the dry weight of mycelium produced in the absence of *P. putida* PMS118S decreased in proportion to the area occupied by the bacterium (Table 4.6 & 4.7).

P. putida PMS118S appears to have affected the allocation of resources to *A. bisporus* hyphae causing the fungus to direct a smaller portion to hyphae growing in the absence of the bacterium. When *A. bisporus* was grown in association with *P. putida* PMS118S the total dry weight of fungal material produced per Petri dish, per unit time, was less than the control (Table 4.7). If biomass was used as an indication of reproductive fitness then this result could be interpreted as an indication that the bacterium was decreasing the overall fitness of the fungus. Biomass, however, does not always provide a good indication of reproductive fitness in fungi (Rayner *et al.* 1985a). It is also unlikely that *P. putida* PMS118S was decreasing the fitness of *A. bisporus*, for if this was occurring then growth of hyphae in the presence of the bacterium would be unlikely, especially when growth could be re-directed toward unexploited regions of the nutritious compost-malt substrate. Furthermore, hyphae of *A. bisporus* do not grow in the presence of bacterial pathogens and hyphal growth is not stimulated by bacteria unable to promote basidiome initiation (see section 4.3.2.).

An alternative and plausible interpretation of this result is that the decrease in the dry weight of fungal material, associated with hyphae growing in the absence of *P. putida* PMS118S , indicates a commitment to reproductive growth. Assuming that *P. putida* triggers the fruiting response in *A. bisporus*, and provided a resource unit of sufficient size to support fruit body production has been exploited (Rayner *et al.* 1984, section 4.4.3), then energy expended on vegetative mycelial growth would reduce the amount of substrate available for reproductive growth (Chanter & Thornley 1978). The fungus would therefore benefit by ceasing to grow in the absence of *P. putida*, once this commitment has been made.

Nutrients absorbed by hyphae growing in the absence of *P. putida* may be either re-directed to hyphae growing in the presence of the bacterium, or stored within the mycelium/mycelial strands, ready to be relocated once initiation of primordia occurs. It would be of considerable interest to determine the concentration and distribution within the mycelium of transport carbohydrates, such as trehalose (Hammond & Nichols 1976), during the preliminary stage of the interaction.

The strategy for reproduction outlined above follows the 'bang-bang' response (Silby & Calow 1986) which is typical of ephemeral species. This strategy would be beneficial for fungi, such as *A. bisporus*, which in the wild inhabit a soil/grassland environment where nutrient sources are temporally and spatially distributed. The ability to switch rapidly and completely, from vegetative to reproductive growth, as soon after exploiting a suitable resource unit as environmental parameters permit, would be an advantage, enhancing the survival potential of the fungus.

The results presented in section 4.3.4. and discussed above were gained from *in vitro* studies and may not necessarily provide a true representation of the situation *in vivo*. Nevertheless, when considered in the light of the results presented in section 4.3.2. and the observations of Eger (1972), Urayama (1967) and Rainey & Cole (1987), they provide strong evidence for the existence of a preliminary reproductive stage in the process of basidiome morphogenesis of *A. bisporus*. This initial reproductive event appears to be concerned with preparing the foundation for subsequent basidiome development. A well laid foundation would enable development of fruit bodies to occur rapidly and would help to maximize the number of basidiomata produced.

CHAPTER FIVE

ISOLATION AND CHARACTERIZATION OF MUTANTS OF *PSEUDOMONAS PUTIDA* ALTERED IN THEIR ABILITY TO PROMOTE BASIDIOME INITIATION OF *AGARICUS BISPORUS* AND DEVELOPMENT OF A GENE CLONING/MAPPING SYSTEM IN *P. PUTIDA*

5.1. INTRODUCTION

5.1.1. GENE TRANSFER IN *PSEUDOMONAS*

The application of genetic engineering technology to any system is dependent upon available gene transfer systems. The three common systems of gene recombination found in other bacterial groups, namely, transformation, transduction and conjugation, have been observed in *Pseudomonas*. These systems are well developed in *P. aeruginosa*, however, in other species, including *P. putida* and *P. fluorescens*, systems enabling the exchange of genetic material, especially among non-laboratory strains are limited. This is largely due to the diversity of these two species and as a result gene transfer systems developed for one species do not usually function in other strains of the same species, let alone other species. (Holloway & Morgan 1986).

Conjugation is the most commonly used method of genetic exchange among pseudomonads and has been used extensively for both gene mapping and gene cloning. Fortunately, many strains, including wild type strains of *P. putida*, will accept broad host range plasmids, such as those of the IncP1 group, which form the basis of many cloning and mapping systems. Conjugation has been most extensively investigated in *P. aeruginosa*, and to some extent the results have been applicable to other members of the genus (Holloway 1986). Plasmids, in particular FP2 and R68.45, which possess chromosome mobilization ability (Cma) (see below), have been used for gene mapping and demonstration of chromosome circularity in *P. aeruginosa* strains PAO (Royle *et al.* 1981) and PAT (Watson & Holloway 1978).

Transduction is not commonly used as a means of genetic exchange, however, bacteriophages can be readily isolated from soil, sewage, or other lysogenic strains and transducing phages have contributed to gene mapping data in several species, including, *P. aeruginosa* (Morgan 1979) and *P. putida* (Chakrabarty *et al.* 1968). Methods for transformation have been developed in laboratory strains of *P. aeruginosa* (Mercer & Loutit 1978) and *P. putida* (Mylroie *et al.* 1978), but most pseudomonads are not transformable and those that are, are transformed at a low frequency (Mermod *et al.* 1986).

5.1.2. GENE CLONING IN *PSEUDOMONAS*

P. aeruginosa is the best known pseudomonad from a genetic perspective because of its role in nosocomial infections and its intrinsic tolerance to many antibiotics (Jacoby 1986). *P. putida* and other pseudomonads have not been so well investigated, but because of the importance of pseudomonads, especially *P. putida* and *P. fluorescens*, in industry and agriculture, considerable effort has recently been directed toward understanding the genetics and biochemistry of these organisms (Mermod *et al.* 1986).

Mutagenesis

Genetic studies require the production of mutant bacteria. Most genes have a spontaneous mutation rate, but this is usually too low for variants to be detected unless an effective selection pressure can be applied. A variety of methods have been used to generate mutants in fluorescent *Pseudomonas* species, including chemical mutagenesis; ethylmethane sulphonate (EMS) (Ankenbauer *et al.* 1986, Kloepper & Schroth 1981) and *N*-methyl-*N'*-nitronitrosoguanidine (NG) (Kloepper & Schroth 1981, Gutterson *et al.* 1986), UV irradiation (Kloepper & Schroth 1981) and transposon mutagenesis (Marugg *et al.* 1985, Morgan & Chatterjee 1985, Anderson & Mills 1985, Cuppels 1986, Poole & Hancock 1986, Sokol 1987, Harayama *et al.* 1984).

Transposon mutagenesis

Transposable genetic elements, or transposons, are used increasingly as a means of generating mutants. Transposons are discrete segments of DNA capable of translocating from one site on a genetic element, to a variety of sites on another (Kleckner *et al.* 1977, see Starlinger 1980). Transpostion of the transposon into a gene usually leads to inactivation of that gene (Kleckner *et al.* 1977) and such mutations are usually non-leaky and stable (de Bruijin & Lupski 1984). Transposition does not require DNA based sequence homology between donor and recipient replicons and occurs independently of RecA-controlled homologous recombination (Foster 1984). Insertion of the transposon into an operon usually exerts polar effects on expression of the genes located downstream from the insertion site (Berg *et al.* 1980).

The *E. coli* derived transposon, Tn*5* (Berg & Berg 1983), which encodes resistance to kanamycin, has a high frequency of transposition and a low specificity of insertion (de Bruijn & Lupski 1984) and is frequently used for mutagenesis in *P. putida*/*P. fluorescens* (Marugg *et al.* 1985, Sykes *et al.* 1985, Harayama *et al.* 1984). Transposon Tn*5* mutagenesis of *Pseudomonas* strains is usually carried out by mobilization of a suicide vector, such as pSUP2021, from an *E. coli* donor strain. The vector pSUP2021 is a derivative of a multi-copy vector pBR325 into which the mobilization site (Mob site or *OriT*) of the broad host range plasmid RP4 has been cloned. The Mob site is the origin of transfer of the IncP1 transfer system which allows mobilization of pSUP2021 in *trans* by IncP1 group plasmids (Simon *et al.* 1984). Replication of the *E. coli* derived vector pBR325 does not occur in other bacteria (Simon *et al.* 1983, Lehrbach & Timmis 1983) and therefore, when introduced into *P. putida* by conjugation, it functions as a suicide vector for the introduction of Tn*5*. Selection of colonies resistant to kanamycin enables identification of transposon carrying clones.

In addition to generating mutants through gene inactivation, transposons have been used in *Pseudomonas* as vehicles to transfer cloned genes into the bacterial chromosome (Grinter 1983). Tn*5* into which the mobilization genes from RP4 have been cloned (Tn*5*-Mob) has been used to mark and mobilize cryptic plasmids in *Rhizobium* (Simon 1984). Tn*5*-Mob is introduced into host genome via a suicide plasmid system (pSUP5011) similar to that described above and host replicons into which Tn*5*-Mob inserts are then mobilizable into other Gram-negative species, provided the transfer functions of plasmid RP4 are available in *trans* (Simon 1984).

Transposons have also been used to provide sites of homology on the bacterial chromosome for conjugative plasmids carrying the same transposon. This enables the formation of effective Hfr donors, that is, bacteria able to transfer their chromosomes in a particular orientation from a unique origin to an appropriate recipient strain, thus allowing mapping of chromosomal genes (Simon *et al.* 1983, Mermod 1986).

Plasmids in *Pseudomonas*

Plasmids are an important component of the genetic make up of pseudomonads. Some, such as FP2 (Holloway & Jennings 1958), act as fertility factors, many confer resistance to antimicrobial

compounds (R-plasmids) (see Jacoby 1986) and some, including CAM (Rheinwald *et al.* 1973), and TOL (Duggleby *et al.* 1977), provide the cell with the ability to degrade recalcitrant compounds. Plasmids are also frequently found in plant pathogenic pseudomonads where they have been associated with toxin and phytohormone production (see Panopoulos & Peet 1985) and Rainey & Cole (1987) produced evidence which suggested the involvement of plasmid DNA in basidiome initiation of *A. bisporus*. A range of cryptic plasmids have also been isolated whose function remains to be determined (see for example Denny 1988).

Pseudomonas plasmids have been effectively classified according to compatibility, that is, by the capacity of a given plasmid to co-exist with other plasmids in the same cell. Plasmids of the various incompatibility groups have different host ranges. The most promiscuous and useful, from a genetic perspective, are the IncP1 plasmids, including RP4, RP1 and RK2. These plasmids are self tansmissible and can be transferred at high frequency to virtually any facultative Gram-negative bacillus (Jacoby 1986). Plasmids of IncP2 include R-plasmids and plasmids associated with the degradation of unusual carbon sources (see Frantz & Chakrabarty 1986). Some of these plasmids are very large, approximately 500 kb (Rheinwald *et al.* 1973), however, the majority of *Pseudomonas* plasmids are between 30 and 100 kb (Jacoby 1986).

Host-vector systems

Unlike *E. coli*, host-vector systems are not well developed in *Pseudomonas* and much cloning work is performed in *E. coli* using the well defined *E. coli* systems. The *E. coli* host-vector systems have several advantages:- The genetic and functional properties of *E. coli* are well defined and experimental conditions, such as those for the isolation of plasmid DNA, are established. A range of well characterized vectors are also available and importantly, cloned *Pseudomonas* genes on a plasmid are unlikely to possess sufficient homology with the *E. coli* chromosome to allow homologous recombination to occur. Cloning of *Pseudomonas* genes in *E. coli* does, however, present some problems and poor expression and instability of many *Pseudomonas* genes is often encountered (Nakazawa & Inouye 1986) demonstrating the need to develop *Pseudomonas* cloning vectors.

Few recombination deficient (Rec$^-$), restriction endonuclease defective, *Pseudomonas* host cells exist into which vector and hybrid DNA molecules can be introduced with high efficiency. Several host strains of *P. aeruginosa* have been developed which are recombination deficient (Chandler & Krishnapillai 1974), or which are deficient in their restriction systems (Wood *et al.* 1981), but no strains with both these characteristics have yet been constructed. Recombination deficient strains are particularly important as recombination between cloned DNA sequences and homologous sequences present in the host, frequently results in loss of the cloned sequences. The frequency of other types of recombination and DNA rearrangements is also reduced in recombinant deficient cells.

Cloning vectors developed for *Pseudomonas* are predominantly of the broad host range type. The best characterized and most frequently used are those based on the IncP4 group plasmids, RSF1010/R1162, such as pKT231 (Bagdasarian *et al.* 1981), and the IncP1 plasmid group RP4/RP1/RK2/R68, such as pLAFR1 (Friedman *et al.* 1982, Moores *et al.* 1984). A frequently used *in vitro* cloning strategy for pseudomonads of the *P. putida/P. fluorescens* group, often referred to as cosmid cloning, involves the generation of a gene bank in *E. coli* using the broad host range cosmid vector pLAFR1 (Friedman *et al.* 1982). *Pseudomonas* DNA is partially digested with *Eco*R1, size-fractionated and ligated to *Eco*R1-digested pLAFR1 DNA. The ligated DNA is then package into lambda phage heads *in vitro* and the phage particles used to transduce *E. coli*. Complementational analysis is then performed by mating the gene bank, either individually, or *en masse*, using helper plasmid pRK2013 (Figurski & Helinski 1979), with the desired mutants. This strategy has been used successfully for cloning of the genes involved in siderophore biosynthesis in *P. putida* (Marugg *et al.* 1985) in cloning of genes involved in the biosynthesis of pseudobactin in a *P. putida/fluorescens* strain (Moores *et al.* 1984) and in cloning of the genetic determinants for inhibition of fungal growth by a fluorescent pseudomonad (Gutterson *et al.* 1986).

Chromosome transfer and prime plasmid formation

The techniques of *in vitro* genetic engineering have permitted the isolation of unusual recombinants containing genetic information from unrelated organisms. The ability of some

plasmids to promote chromosome transfer between different bacterial genera provides an alternative set of procedures for achieving such unusual recombinants and is frequently referred to as *in vivo* genetic engineering (Holloway 1979).

Genetic studies of processes and pathways in bacteria usually require replacement of one allele of a gene by another allele, or the construction of strains merodiploid for a given region of the chromosome (Van Gijsegem & Toussaint 1982). Manipulations of this kind can be performed using plasmids able to mobilize the bacterial chromosome. The best known plasmid with this ability is the *E coli* sex plasmid F which is able to integrate stably into the bacterial chromosome at a variety of sites to create strains which are conjugational donors of chromosomal markers at high frequency (Hfr strains) (see Low 1972). In *Pseudomonas*, several plasmids exist which possess chromosome mobilization ability (Cma). The FP plasmids of *P. aeruginosa*, especially FP2, have been used extensively for introduction of genes into other strains and gene mapping, however, they have a limited number of insertion sites within the *P. aeruginosa* chromosome and a narrow host range.

The best known *Pseudomonas* plasmid with Cma is derived from R68, a broad host range plasmid, similar to the widely studied RP1 and RP4. R68 was shown by Stanisich & Holloway (1971) to mobilize the chromosome in *P. aeruginosa,* although it was bacterial strain specific. Variants of R68 were sought which would mobilize chromosome in other *P. aeruginosa* strains. A derivative, R68.45, containing a duplicated insertion sequence, IS*21* (Willets *et al.* 1981), was found which was able to mobilize the chromosome in a wide range of bacteria including *P. aeruginosa* (Haas & Holloway 1976), *P. putida* (Holloway 1978), *R. leguminosarum* (Beringer & Hopwood 1976) and *Agrobacterium tumefaciens* (Hamada *et al.* 1979). R68.45 and plasmids like it, are known as enhanced chromosome-mobilizing (ECM) plasmids. These plasmids are able to mobilize chromosome because they possess a transposable sequence (R68.45 possess the transposable sequence IS*21*) which, during transposition from chromosome to plasmid, forms a cointegrate intermediate between the two replicons (Foster 1984). As a result of the non-specific nature of the transposition process, R68.45 displays a multiplicity of transfer origins

which has greatly aided linkage analysis of bacterial chromosomes, demonstration of chromosome circularity and intergeneric crosses (Holloway 1978, 1986).

Most plasmids with Cma are also able to form prime plasmids (Holloway & Low 1987), that is, plasmids into which fragments of chromosomal DNA have become incorporated. Prime plasmids were first detected in *E. coli* where they were formed as a result of the incorporation of a fragment of chromosome into the sex plasmid F (Low 1972). Such F plasmid-chromosome hybrids are known as F-prime plasmids, similarly, prime plasmids formed by R-plasmids are known as R-prime plasmids. R68.45, like the F plasmid is able to promote the formation of R-prime plasmids, and these plasmids retain the wide bacterial host range of the parent plasmid enabling the transfer of bacterial genes between genera. This enables their expression to be studied and provides a means of constructing bacterial hybrids with novel characteristics (Holloway & Low 1987).

The mechanism of prime plasmid formation is not well understood, but are thought to form as a result of the interaction between identical sequences on the plasmid and the host chromosome (Holloway & Low 1987). Van Gijsegem & Toussaint (1982) proposed a mechanism for the formation of R-primes with RP4::mini-Mu and this is shown in Fig.5.1 (RP4::mini-Mu is discussed in more detail below).

Prime plasmids have been generated by R68.45 in a range of bacteria, including *P. aeruginosa* (Holloway 1978, Morgan 1982), *Rhizobium leguminosarum* (Johnstone *et al.* 1978) and *Klebsiella pneumoniae* (Espin *et al.* 1981). Prime plasmids have been formed in *P. putida* using R68.44 (Beeching *et al.* 1983), a plasmid derived from R68.45 and R91-5::Tn*501* (Cain & Holloway 1984). In addition to enabling the formation of interspecific and intergeneric hybrids, prime plasmids have also been used for mapping in *P. aeruginosa* (Tsuda *et al.* 1981) and for identifying biochemical lesions in *P. putida* by complementation (Morgan 1982, Morgan & Dean 1985). Prime plasmids have also been used to isolate sections of chromosomal DNA from which selected genes have been cloned by *in vitro* methods ((Van Gijsegem *et al.* 1985).

Prime plasmids generated by plasmids such as R68.45 have been isolated in two ways. The first involves mating a donor, carrying R68.45, with a recombination deficient recipient with a

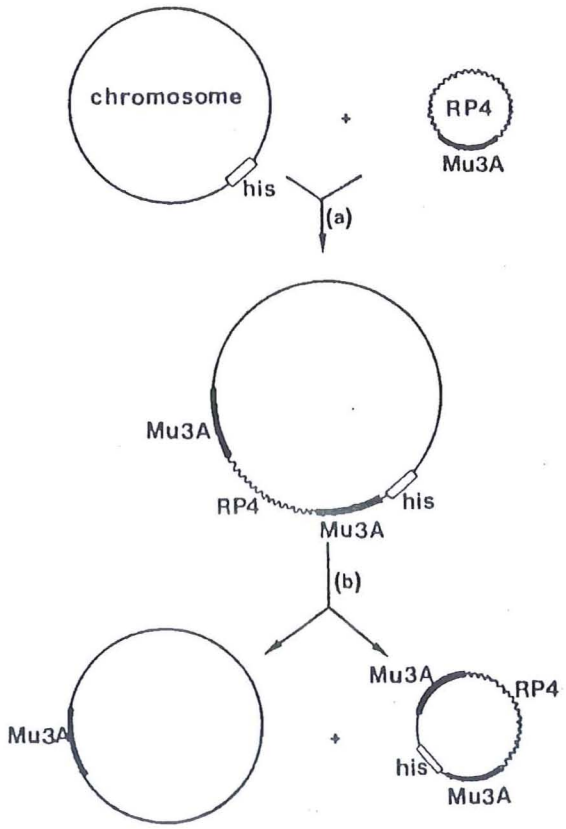


Fig. 5.1. Proposed mechanism for the formation of R-primes with an PR4::mini-Mu (after Van Gijsegem & Toussaint 1982). (a) Mini-Mu mediated integration of the RP4::mini-Mu near the *his* locus. The RP4 is flanked by two copies of mini-Mu in the same orientation (direct repeat). (b) Mini-Mu mediated formation of an R-prime *his*. The mini-Mu prophage distal to the *his* marker mediates formation of a deletion of the RP4, the mini-Mu proximal to the *his* marker, and adjacent host DNA, including the *his* locus. This leads to the formation of an R-prime mini-Mu-*his*-mini-Mu plasmid. The mini-Mu prophage distal to the *his* marker is duplicated in the process, so that a mini-Mu copy remains in the chromosome at the site where the deletion occurs.

selectable marker such as an auxotrophic marker (Holloway 1978). Prototrophic recombinants for this marker can arise only by transfer to the recipient of a transmissible plasmid carrying a segment of the donor chromosome with this wild type allele, because integration of the region carrying that allele into the recipient chromosome is prevented by the recombination-deficient phenotype (Holloway 1978).

The second procedure is used when recombination-deficient strains are not available and involves mating a donor carrying R68.45 with a recipient strain belonging to a different genera. By using different species, or genera, there should be sufficient non-homology to ensure that the cloned sequences are not lost by reciprocal recombination between homologous fragments. R-prime plasmids have been isolated in *P. putida* using this method (Beeching *et al.* 1983)

***In vivo* cloning using RP4::mini-Mu**

Another plasmid derived from the IncP1 group plasmids, RP4::mini-Mu, has been used to successfully promote chromosome transfer and generate R-prime plasmids in a wide range of bacterial genera. This plasmid is based on the broad host range plasmid RP4 and carries a deleted Mu prophage, Mu3A (Van Gijsegem & Toussaint 1982). RP4 has been shown to mobilize the chromosome of *Acinetobacter calcoaceticus* (Towner & Vivian 1977) and *Caulobacter crescentus* (Barret *et al.* 1982), but not the chromosome of genera such as *Pseudomonas*, *Rhizobium*, *Agrobacterium* or *Escherichia*. However, various RP4 derivatives constructed *in vitro* have been shown to transfer the chromosome of *E. coli* (Barth 1979), *Proteus morganii* (Beck *et al.* 1982) and *Rhizobium trifolii* (Kowalczuk *et al.* 1981).

Bacteriophage Mu is a giant transposon under the cloak of a virus (see Bukhari 1976) and exhibits great recombinational versatility. Unlike other temperate phages, Mu integrates into the host genome when it enters the lytic cycle, or the lysogenic state. Moreover, this integration is a random event. During the lytic cycle Mu transposes to new locations on the host chromosome and generates chromosomal rearrangements including deletions, inversions, duplications and transpositions of host DNA segments, and fuses replicons, such as two plasmids, or a plasmid and a chromosome (see Toussaint & Resibois 1983). These transpositional properties render Mu a very useful genetic tool.

One consequence of these properties is that the presence of Mu in a plasmid increases its potential to interact with other replicons (Desmet *et al.* 1981). IncP1 plasmids, such as RP4, containing Mu have been shown to promote chromosome transfer in *E. coli* and *Klebsiella pneumoniae* strains made lysogenic for Mu (Murooka *et al.* 1981), strains in which RP4 alone, will not promote chromosome transfer. There are, however, disadvantages in using intact Mu to promote chromosome transfer and generate R-prime plasmids. These occur because of the necessity to integrate Mu into the chromosome of the donor strain and then to use a recipient strain which is (i) lysogenic for Mu (to avoid zygotic induction) and (ii) resistant to Mu (to avoid reinfection and killing of the recipient by the phages released upon induction of the donor) (Van Gijsegem & Toussaint 1982). Furthermore, the efficiency of transfer of RP4::Mu into a nonlysogenic, heterospecific recipient is usually low, probably due to the combined effects of restriction of foreign DNA and zygotic induction (Lejeune *et al.* 1983).

In an attempt to overcome these problems mini-Mu derivatives carrying large internal deletions were isolated by Faelen *et al.* (1978). These mini-Mu prophages, such as Mu3A, are deleted for all of the Mu lethal functions (Faelen *et al.* 1978, Resibois *et al.* 1981), but contain a functional Mu gene *A* and intact ends of the Mu genome which are required for maintenance of the transposition related properties (Faelen *et al.* 1978). Van Gijsegem & Toussaint (1982) introduced Mu3A into RP4 and using this RP4::mini-Mu plasmid were able to promote chromosome transfer and R-prime formation, at high frequencies (10^{-4} per recipient cell for the transfer of a given marker, 10^{-5} for the formation of R-primes carrying a given marker) in *E. coli*, *Salmonella typhimurium, K. pneumoniae* and *P. mirabilis*, in both homologous and heterologous matings. Since this work was published, RP4::mini-Mu plasmids have been used to promote chromosome mobilization and R-prime formation in a wide range of bacteria including *P. fluorescens, Alcaligenes eutrophus* (Lejeune *et al.* 1983), *Erwinia amylvora, E. chrysanthemi* and *E. carotovora* (Chatterjee *et al.* 1985). RP4::mini-Mu plasmids, especially pULB113 (Van Gijsegem & Toussaint 1982), have also been used to construct a genomic map of *Aeromonas hydrophila* (Gobius & Pemberton 1986) to clone genes in *E. carotovora* involved in catabolism of hexuronates (Van Gijsegem & Toussaint 1983), to clone the pectate lyase and cellulase genes of

E. chrysanthemi (Van Gijsegem *et al.* 1985) and to identify genes involved in siderophore biosynthesis in *E. chrysanthemi* (Enard *et al.* 1988)

5.1.3. STUDY AIMS

The aim of this study was to isolate and characterize mutants which were defective in their ability to promote basidiome initiation. Provided this was successful, then it would be possible to isolate, clone and map the genes involved in the process of basidiome initiation. For this purpose the RP4::mini-Mu *in vivo* cloning and mapping system was developed in *P. putida*, a species in which the potential of RP4::mini-Mu has not been investigated.

5.2. MATERIALS AND METHODS

5.2.1. BACTERIAL STRAINS AND MEDIA

Bacterial strains, plasmids, bacteriophages and DNA fragment size standards used in this study are described and listed in Table 5.1. All strains were grown in Luria (L) broth. *E. coli* and *P. aeruginosa* strains were incubated at 37 °C and *P. putida*, *P. tolaasii* and *Serratia entomophila* strains were incubated at 28 °C. For longterm storage, broth cultures were grown to stationary phase, mixed with 20 % glycerol and stored at -80 °C. *Pseudomonas* strains were also stored in 1/50 strength KB broth (2.3.4.) at room temperature. King's Medium B (KB) and L-agar were used in routine sub-culturing of strains. Concentrations of antibiotics used in selective media are presented in Table 5.2. Stock solutions of antibiotics were filter sterilized (0.22 μm, Millipore) and stored in aliquots at -20 °C.

5.2.2. MUTAGENESIS

Ultraviolet irradiation (UV)

Ultraviolet (UV) light (254 nm) is an effective mutagen which causes both base substitutions and deletions (Miller 1972).

Bacteria to be mutagenized were grown overnight in 5 ml of L-broth, spun down and resuspended in 5 ml of 0.1 M $MgSO_4.7H_2O$ to a density of 2-3 x 10^8 cells ml^{-1}. The cell suspension was poured into a 90 mm diam glass petri dish and placed below an UV light source (Miller 1972). UV mutagenesis was conducted at two different institutes and consequently, different UV lights were used; at I.H.R. the UV light was 0.35 m above the bench, while at Canterbury University it was 0.55 m above the bench. The UV light at Canterbury University was calibrated using a dosimeter.

Survival curves for each UV light source were determined by removing samples (0.1 ml) from the irradiated dish every 10 s. These were titred immediately on nutrient agar/yeast extract plates. The survival curves enabled determination of the length of time necessary to expose the cells to the mutagen to ensure survival of between 0.01 and 0.1 % of the cells. All

Table 5.1. Bacterial strains, plasmids, phages and DNA fragment size standards

Strain	Characteristics	Reference/source
P. putida		
PMS118	pPU1 pPU2 Am Nx Cm Em (Tc^S Km^S Sm^S Rm^S) Bas^+	This study
PRP118	PMS118(RP4)	PMS118 x HB101(RP4)
PGT(3)118	PMS118(pULB113)	PMS118 x MXR(pULB113)
PGT(2)118	PMS118(pULB21)	PMS118 x MXR(pULB21)
PGT(1)118	PMS118(pULB110)	PMS118 x CSH57(pULB110)
PMS195	plasmid$^-$ Am Nx Cm Em (Tc^S Km^S Sm^S Rm^S) Bas^+	This study
PMS196	plasmid$^-$ Am Nx Cm Em Tc (Km^S Sm^S Rm^S) Bas^+	This study
PRP196	PMS196(RP4)	PMS196 x HB101(RP4)
PGT(3)196	PMS196(pULB113)	PMS196 x MXR(pULB113)
PGT(2)196	PMS196(pULB21)	PMS196 x MXR(pULB21)
PGT(1)196	PMS196(pULB110)	PMS196 x CSH57(pULB110)
PPU196	PMS196(pPU1)	PMS196 x PMS118 (pPU1::Tn5-Mob)(pJB3JI)
PMS234	Am Nx Cm (Tc^S Km^S Sm^S Rm^S Em^S) Bas^+	This study
P. tolaasii		
PMS117	pTO1 Am Em Nx (Cm^S Km^S Sm^S Tc^S)	This study
PGT(3)117	PMS117(pULB113)	PMS117 x MXR(pULB113)
PGT(2)117	PMS117(pULB21)	PMS117 x MXR(pULB21)
Serratia entomophila		
IS1	plasmid$^-$ Sm (Km^S)	
IPUS1	IS1(pPU1)	IS1 x PMS118(pPU1::Tn5-Mob)(pJB3JI)
P. aeruginosa (PAO)		
OT11	*leu-1 pro-1* Sm (Km^S) plasmid$^-$	I. Lamont, Dept. of Biochemistry, University of Otago
OTGT(3)11	OT11(pULB113)	OT11 x PMS118(pULB113)
OTGT(2)11	OT11(pULB21)	OT11 x PMS118(pULB21)
PAO2003	*arg-32 str-39 chl-2 rec-2* (Km^S) plasmid$^-$	Chandler & Krishnapillai (1974)
PAOGT(3)2003	PAO2003(pULB113)	PAO2003 x PMS118(pULB113)
OT899	*leu-1 metF26*::Tn801	B. Palmer, Dept. of Microbiology, University of Otago
E. coli (K12)		
HB101	*leuB6 proA2 ara-14 xyl-5 galK2 mtl-1 lacY1 thi hsdS20 supE44 recA13 rpsL20*	D. Hill, dept. of Biochemistry University of Otago
HBPU101	HB101(pPU1)	HB101 x PMS118(pPU1::Tn5-Mob)(pJB3JI)
HBRP101	HB101(RP4)	HB101 x OT899(RP4)

Table 5.1 cont.

Strain		Characteristics	Reference/source
HBGT(3)101		HB101(pULB113)	HB101 x PMS118(pULB113)
HBGT(2)101		HB101(pULB21)	HB101 x PMS118(pULB21)
S17-1		*pro hsdR* RP4-2 (Tc::Mu)(Km::Tn7)	Simon *et al.* (1984)
RR1		F⁻ lambda⁻ *hsd20 ara14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*	Bolivar *et al.* (1977)
MXR		F⁻ (*lac-pro*) *thi recA-1 galE leu purE trp his argG ilv met mtl gal lacY xyl ara malts rpsL*	Faelen *et al.* (1978)
CSH57			Miller (1972)
Plasmids	**Host**		
pULB113	MXR	RP4::Mu3A (mini-Mu); Tra⁺, Am, Km, Tc	Van Gijsegem & Toussaint (1982)
pULB21	MXR	RP4 Tc::Mu3A (mini-Mu); Tra⁺, Am, Km	Van Gijsegem & Toussaint (1982)
pULB110	CSH57	RP4 Km::Mu3A (mini-Mu); Tra⁺, Am, Tc	Laboratoire de Genetique, Universite Libre de Bruxelles
RP4	OT899	IncP1; Tra⁺, Km, Am, Tc	Datta *et al.* (1971)
RP1	PAO	IncP1; Tra⁺, Km, Am, Tc	Ingram *et al.* (1973)
pJB3JI	AK1072	Derivative of R68.45; Tra+, Am, Tc, (Km^s)	D. B. Scott, Dept. of Microbiology and Genetics, Massey University
pSUP2021	SM10	(pBR325-Mob)::Tn5; Km, Cm, Am, Tc	Simon *et al.* (1984)
pSUP5011	S17-1	(pBR325)::Tn5-Mob; Km, Cm, Am	Simon (1984)
pPU1	PMS118	cryptic	This study
pPU2	PMS118	cryptic	This study
pTO1	PMS117	cryptic	This study
Phages			
PR4		IncP1 pili specific lytic phage	Bradley & Cohen (1977)
Mu*cts*62		Thermoinducible Mu phage	Howe (1973)
Fragment size strandards			
Lambda ladder		*Hin*dIII digested lambda DNA; 23.6, 9.64, 6.64, 4.34, 2.26 and 1.98 kb fragments	BRL
pSAC3		7.6 kb	G. T. Timmerman, Dept. of Plant & Microbial Sciences, University of Canterbury
RP4		60 kb	Datta *et al.* 1971
FP2		90 kb	Holloway & Jennings (1958)

operations carried out after exposure to UV light were conducted in the dark and cells were incubated in the absence of light.

Table 5.2. Antibiotic concentrations used in selective media

	Concentration (μg ml^{-1})	
Antibiotic	*P. putida/E. coli*	*P. aeruginosa*
Ampicillin (Am)	50	-
Chloramphenicol (Cm)	50	-
Erythromycin (Em)	100	-
Kanamycin (Km)	50	500
Naladixic acid (Nx)	20	-
Rifampicin (Rm)	100	-
Streptomycin (Sm)	100	200
Tetracycline (Tc)	20	-

Chemical mutagenesis

Ethylmethane sulphonate (EMS)

EMS, like nitrosoguanidine, is a potent mutagen which causes transversions and transitions to occur, and is useful for the production auxotrophic mutants (Carlton & Brown 1981). EMS was used by Watson & Holloway (1976) to produce suppressor mutations in *P. aeruginosa*. The method described by Miller (1972) was used.

Cells were grown to 2-3 x 10^8 cells ml^{-1} in 4 ml of glucose M63 medium, spun down, washed and resuspended in half the original volume of minimal medium containing 0.2 M Tris (pH 7.5), but no carbon source. To this was added 30 μl EMS and the samples shaken vigorously for 2 h at 28 °C before spreading appropriate dilutions onto L-agar plates. The survival rate was determined by titrating the culture before and after mutagenesis. Colonies from mutagenized cultures with survival rates between 0.1 and 0.01 % were used.

Nitrous acid

Nitrous acid produces bidirectional transition mutations and deletions (Carlton & Brown 1981) and was used by Kerppola & Kahn (1988) for the production of auxotrophic mutants of *R. meliloti*. The method described by Carlton & Brown (1981) was used.

The culture to be mutagenized was grown overnight in 5 ml L-broth, spun down and washed in 5 ml 0.1 M sodium acetate buffer (pH 4.6). A fresh solution of sodium nitrite was prepared (0.05, 0.005, and 0.0005 M) in 0.1 M sodium acetate buffer, pH 4.6). The cells were resuspended in 1 ml of the nitrous acid solution and incubated at 28 °C. Aliquots (0.1 ml) were removed after 0.5, 1, 2, 5, 10 and 15 min incubation and added to 10 ml M63 medium to stop the reaction. Appropriate dilutions were spread on L-agar plates.

Transposon mutagenesis

Transposon Tn*5* mutagenesis was carried out as described by Simon *et al.* 1983, and some of the modifications of Marugg *et al.* 1985 were incorporated. Cells of *E. coli* donor S17-1 containing pSUP2021 (sometimes pSUP5011) and *Pseudomonas* recipients, were grown overnight in 5 ml L-broth and 0.1 of donor and recipient were mixed in an Eppendorf tube and concentrated by a short centrifugation (10 s). The mating mixture was carefully suspended in 50 μl of prewarmed L-broth and spread on a 14 mm diam Millipore nitrocellulose filter (0.45 μm pore size) which was placed on a prewarmed L-agar plate. The cells were left for 4 h at 28 °C and then suspended in 1 ml of L-broth and 0.1 ml aliquots spread onto either KB, KB containing 100 μM $FeCl_3$, or L-agar, all of which contained naladixic acid (to select against the donor) and kanamycin (to select against the recipient). Once transconjugant colonies had reached a diameter of 2 - 3 mm (3 d) they were removed with sterile tooth picks and patched onto fresh selective media before determining their phenotype.

5.2.3. MUTANT CHARACTERIZATION

Selection of rifampicin resistant mutants

P. putida strains PMS118S and PMS196 were grown overnight in L-broth and 100 μl spread on plates of L-agar containing 100 μg ml^{-1} rifampicin. The plates were incubated for 24 h before selection of rifampicin resistant colonies. Clones having growth and colony characteristics similar to the wild type were tested for their ability to stimulate mycelial growth, promote basidiome initiation and level of antibiotic resistance.

Characterization of recombination deficient strains (Rec⁻)

A recombination deficient *P. putida* PMS118S mutant was sought so as to enable formation of stable R-prime plasmids in homologous matings between P. putida strains containing RP4::mini-Mu. In experiments aimed at cloning the genes responsible for promoting basidiome initiation, it would be necessary, in order to maintain these genes on an RP4::mini-Mu plasmid, to perform homologous matings between appropriate mutants (in a Rec⁻ background) and the wild type strain containing an RP4::mini-Mu plasmid. Failure to use a recombination deficient recipient would enable recombination to occur between the two similarly orientated mini-Mu sequences on the plasmid (a result of the transposition process - see Fig. 5.1.) which would result in excision of the cloned genetic material (Toussaint *et al.* 1981). It would not have been feasible to generate R-prime plasmids by performing heterospecific crosses, as previous work had shown that *Pseudomonas* species other than *P. putida* are rarely able to promote basidiome initiation.

Construction of Rec⁻ strains is time consuming and usually requires screening for the inability of cells to produce recombinants (see for example Clark & Margulies (1965) or Watson & Holloway (1976)). These methods rely on the presence of well developed recombinational systems, such as the *E. coli* Hfr strains, which do not exist in *P. putida*. Consequently, recombination deficient cells were selected by screening for sensitivity to UV light.

A rifampicin resistant *P. putida* PMS118S mutant (PR216) was irraditated with UV light (transposon mutagenesis was avoided as it was important that this strain retain the kanamycin and streptomycin sensitivity of the wild type (Tn5 expresses both kanamycin and streptomycin resistance in nonenteric bacteria (de Vos *et al.* 1984))) and grown on L agar for 2 days. Colonies were then replica plated onto L-agar plates and immediately exposed to UV light for 30 s. Colonies which failed to grow were examined further and the most UV sensitive mutant was used as a potentially recombination deficient strain.

Characterization of auxotrophs

Ampicilin enrichment for auxotrophs

Cells treated with EMS were subject to ampicillin enrichment using the method described by Miller (1972). After mutagenesis *P. putida* PMS118 cells were grown overnight in M63 minimal broth supplemented with 100 μg ml^{-1} of a selected amino acid. The cells were spun and washed twice in PBS before inoculating M63 broth with approximately 1 x 10^{7} cells ml^{-1}. The cells were allowed to grow for 4 h, or until the turbidity had tripled and then ampicillin (750 μg ml^{-1}) was added and the culture shaken for a further 2 h. Once the cells had lysed, 500 μl of the suspension was removed and added to 10 ml fresh M63 containing the selected amino acid. Following overnight growth and dilution, 0.1 ml aliquots were spread onto M63 supplemented with the appropriate amino acid (40 μg ml^{-1}). Once colonies had reached a diameter of 2 mm they were replica plated onto unsupplemented M63 to enable detection of auxotrophs.

To determine the nutritional requirements of the auxotrophs, individual colonies were replica plated onto supplemented M9 plates containing the eleven nutritional pools described by Davis *et al*. (1980).

Isolation of mutants defective in their ability to promote basidiome initiation

Preliminary mutagenesis experiments using UV light were conducted on *P. putida* PMS118 at I.H.R.. An unusually high proportion of the cells surviving after this treatment failed to produce a fluorescent pigment when plated on KB and several of these non-fluorescent mutants also failed to promote mycelial growth or basidiocarp initiation when plated alongside cultures of *A. bisporus* and W19. These results suggested that the bacterial iron acquiringsystem was involved in the process of basidiome initiation. Consequently, bacteria possessing single gene mutations within their siderophore/iron uptake system were sought.

Characterization of mutants defective in siderophore biosynthesis and ferric-siderophore uptake

Cells treated with either UV light, EMS or subject to transposon Tn*5* mutagenesis were replica plated from KB containing 100 μM $FeCl_3$ onto KB to check for loss of fluorescence (Flu^{-}) and KB

supplemented with 0.31, 0.62, 1.25 and 5.0 mg ml^{-1} EDDA (a synthetic iron chelating agent) to check for their ability to grow under conditions of iron limitation (Vandenbergh *et al.* 1983). Cells unable to grow under iron limitation were inoculated alongside wild type PMS118S on KB supplemented with EDDA to determine there ability to utilize the siderophore secreted by the parent strain. Cells able to grow when provided with the wildtype siderophore were siderophore mutants (Sid^-) and those unable to utilize the wild type siderophore were iron uptake mutants (Iup^-).

Characterization of mutants defective in their ability to promote basidiome initiation

The effect of mutants generated by UV light, EMS, or transposon Tn5 mutagenesis on basidiome initiation and mycelial growth was determined by screening against both *A. bisporus* (strain U3) and *A. bitorquis* (strain W19) (see section 4.2.3.).

5.2.4. DEVELOPMENT OF THE RP4::MINI-MU *IN VIVO* GENE CLONING AND MAPPING SYSTEM IN *P. PUTIDA*

Matings

Matings between RP4 or RP4::mini-Mu-carrying donors and recipients were performed by plating 50 μl of donor and recipient from overnight cultures on 24 h plates of L-agar. The mating mixture was left overnight at 34 °C for matings between PMS118S and *E. coli* or *P. aeruginosa* and 37 °C for matings between PMS196 and *E. coli* or *P. aeruginosa*. Matings between *P. putida* strains were conducted at 28 °C. Bacteria were collected from the plates and suspended in 1 ml of 0.01 M $MgSO_4$, and spread on appropriate selective media. Strains carrying RP4 or RP4::mini-Mu were selected on media containing tetracycline and kanamycin (ampicillin was included if the recipient was an *E. coli* strain). Antibiotics used to select against the donor strain are given in the footnotes to Tables 5.7 and 5.8. Transconjugants prototrophic for one marker were selected on minimal M9 media supplemented with all the nutrients required for growth of the recipient strain, except for that corresponding to the selected marker.

Determination of the presence of RP4 and RP4::mini-Mu

The presence of RP4 and RP4::mini-Mu was checked by testing for resistance to tetracycline and kanamycin (and ampicillin if the recipient was *E. coli*) and sensitivity to phage PR4 (Stanisich 1974, Barth *et al.* 1978), a phage which grows only on strains carrying RP4 or other IncP group plasmids. The presence of mini-Mu was tested for by re-transfering RP4::mini-Mu to *E. coli* and examining for the expression of Mu immunity. This was done according to the method of Van Gijsegem & Toussaint (1982). An overnight culture of the strain to be tested was plated with 3 ml of soft L-agar on a plate of L-agar. Serial dilutions of Mu*cts*62 were spotted on the lawn and the plates incubated at 30 °C and 42 °C. Thermoinducible Mu phage forms plaques at 42 °C but not at 30 °C on strains lysogenic for mini-Mu carrying the *cts* 62 allele. The integrity of the plasmid was also confirmed by restriction endonuclease analysis

Preparation of bacteriophage lysates

P. aeruginosa OT899 containing RP4 was grown overnight in L-broth and then subcultured (0.1 ml) into 5 ml of fresh, prewarmed L-broth. A single plaque removed from an L-agar plate on which a lawn of OT899(RP4) had been previously spread and spotted with serial dilutions of the PR4 lysate was added, and the bacterium and phage mixture shaken for a further 2 h. Three drops of chloroform were then added and following lysis, the cells were spun for 1 min in a bench top centrifuge. The supernatant containing PR4 phage was removed, titred and stored at 4 °C over a drop of chloroform.

A lysate of Mu*cts*62 was obtained by growing *E. coli* strain Q1 harbouring the phage at 30 °C in an orbital shaker until the logorithmic phase of growth was reached. The temperature of incubation was then increased to 42 °C and cell lysis was observed by a decrease in turbidity. Chloroform was added and the lysate treated as described above for PR4.

Isolation of plasmid DNA

Plasmid DNA was isolated according to the method of Birnboim & Doly (1979) using some of the modifications of Birnboim (1983) as outlined below. Cells were grown overnight in 2 ml of L-broth and 0.5 ml placed in a 1.5 ml Eppendorf tube and centrifuged for 15 s in a bench-top

centrifuge at room temperature. The supernatant was removed by aspiration and the pellet dislodged by clicking two tubes together in a vortex mixer. Lysis buffer (Appendix B) was then added (100 μl, 1 mg ml^{-1} lysozyme), the cells quickly suspended by vortex-mixing and placed on ice for 5 min. Alkaline SDS (Appendix B) was gently added (200 μl) and the cells mixed carefully by inverting the tube 2 or 3 times before placin back on ice for a further 5 min. Sodium acetate (pH 4.8) was then added (150 μl) and the tube once again gently mixed by carefully inverting. The tube was returned to the ice for 15 min, spun for 8 min and then 400 μl of supernatant was removed and placed into a fresh Eppendorf tube to which 1 ml of cold (-20 °C) ethanol was added. Precipitation of DNA was aided by placing the tube in a -80 °C freezer for 15 min. The DNA was collected on the side of the tube by centrifuging for 2 min, the supernatant removed and the DNA dried under vacuum. The DNA was resuspended in 100 μl of neutralization buffer (Appendix B) and 200 μl of cold ethanol was added and the tube placed back in the -80 °C freezer for 5 min before pouring off the supernatant and drying the DNA under vacuum.

Additional purification of DNA from *Pseudomonas* cells was sometimes required to remove cell wall carbohydrate material. A large proportion of this was removed by vortexing the DNA with a few drops of chloroform after removal of 400 μl supernatant following addition of sodium acetate. An extra alkali denaturation step was also sometimes used with *Pseudomonas* DNA to remove traces of chromosomal DNA (Birnboim 1983).

Treatment of DNA samples with ribonuclease A was avoided as preliminary experiments demonstrated that when this was added DNA was completely degraded after restriction endonuclease digestion. This was probably due to activation of trace amounts of endonuclease I by ribonuclease A through removal of tRNA, a powerful inhibitor of this enzyme (Lehman *et al.* 1962).

Restriction endonuclease digestion

*Pst*I was obtained from BRL and DNA samples were restricted in suitable buffers according to the manufacturers instructions.

Agarose gel electrophoresis

Electrophoresis of DNA through agarose gels was carried out in TAE buffer (Appendix B). For routine analysis a mini-horizontal gel system was used. Samples (15 μl) were run in 65 x 100 x 5 mm gels, 0.65 % agarose at 80 V, for 50 min. Analytical gels and were also run in a horizontal system. Samples (25 μl) were run in 130 x 140 x 5mm gels, 0.7 % agarose at 80 V, 30 mA for 3 - 4 h. All samples were prepared by the addition of 20 % sample dye prior to electrophoresis (Appendix B). Gels were stained with ethidium bromide (1 μg ml^{-1}), destained in running buffer and DNA visualized on a UV trans-illuminator and photographed.

5.2.5. MARKING AND MOBILIZATION OF A CRYPTIC *P. PUTIDA* PLASMID

Rainey & Cole (1987) produced evidence which suggested that a plasmid was involved in the process of basidiome initiation, however, no differentiation was made between chromosome and plasmid DNA in this study as the RP4::mini-Mu system would allow identification of genes irrespective of their location. Nevertheless, the Tn*5*-Mob system described by Simon (1984) was investigated as a means of marking and mobilzing the *P. putida* PMS118S plasmids. Marking of the plasmids with Tn*5*-Mob would enable their mobilization to strains unable to promote basidiome initiation, or cured, and their role in fruiting easily determined. The Tn*5*-Mob system (Simon 1984) was used and the suicide vector pSUP5011 containing Tn*5*-Mob was introduced into *P. puitda* PMS118S, using the method described above for Tn*5*, except selection was on KB containing 75 μg ml^{-1} kanamycin and 20 μg ml^{-1} naladixic acid. A high concentration of kanamycin was used to increase the probability of finding Tn*5* transpositions into plasmid DNA. Initially 30 kanamycin resistant Tn*5*-Mob carrying *P. putida* PMS118S clones were selected and individually mated with *E. coli* strain AK1072 containing pJB3JI, a kanamycin senesitive R68.45 derivative. Transconjugants were selected on L-agar containing kanamycin and tetracycline and then individually mated with *E. coli* HB101; the donor was selected against with kanamycin and the recipient with streptomycin. In a second experiment 100 PMS118::Tn*5*-Mob clones were selected and mated *en masse* with pJB3JI, selecting for transconjugants as described above. These transconjugants were then mated *en masse* with PR220, a rifampicin resistant, plasmidless strain of *P. putida* PMS196. Transconjugants were selected on KB containing rifampicin and

kanamycin. The presence of plasmid DNA in PR220 was checked by preparing the DNA as described above and the host range of the plasmid was investigated by transfering it from PR220 to *Serratia entompohila* IS1 and *E. coli* HB101 with the help of pJB3JI. Strains containing pPU1 were screened against *A. bitorquis* W19 to determine its effect on basidiome initiation.

5.3. RESULTS

5.3.1. MUTAGENESIS OF *P. PUTIDA*

Rifampicin resistant mutants

Rifampicin resistant colonies of PMS118S and PMS196S were produced at a low frequency ($< 10^{-9}$). Three clones of PMS118 (PR215, PR216 and PR217) and one of PMS196 (PR220) were selected and shown to be similar to the wild type in all respects, except for antibiotic resistance.

Recombination deficient mutants (Rec^-)

Five hundred *P. putida* PR216 UV irradiated colonies were screened for their resistance to UV light. Four colonies were found which displayed a decreased resistance to UV light and one, designated PR118, was found which was extremely UV sensitive. Fig. 5.2 shows the sensitivity of PR118 to UV light.

Auxotrophic mutants

Auxotrophic mutants are shown in Table 5.3. Nearly 50 % of the auxotrophs generated by Tn*5*-Mob were histidine requiring mutants and of nine Tn*5* generated mutants, three were tryptophan auxotrophs. This suggests that there may be preferred sites for insertion of Tn*5* within the *P. putida* PMS118S genome.

Basidiome inhibitory mutants

P. putida PMS118S was initially subject to UV mutagenesis at I.H.R., primarily for the purpose of investigating colony morphogenesis (the survival curve for this light source is shown in Chapter 2.0., Fig. 2.14)). Only a small number of mutants were generated and as a matter of interest, some of the UV irradiated colonies were plated alongside 6 d *A. bisporus* cultures. From fifty mutagenized colonies, several were found which failed to promote mycelial extension. Plating of these mutants onto KB revealed their inability to produce a yellow-green fluorescent siderophore when grown under iron deplete conditions. This fortuitous finding suggested the involvement of

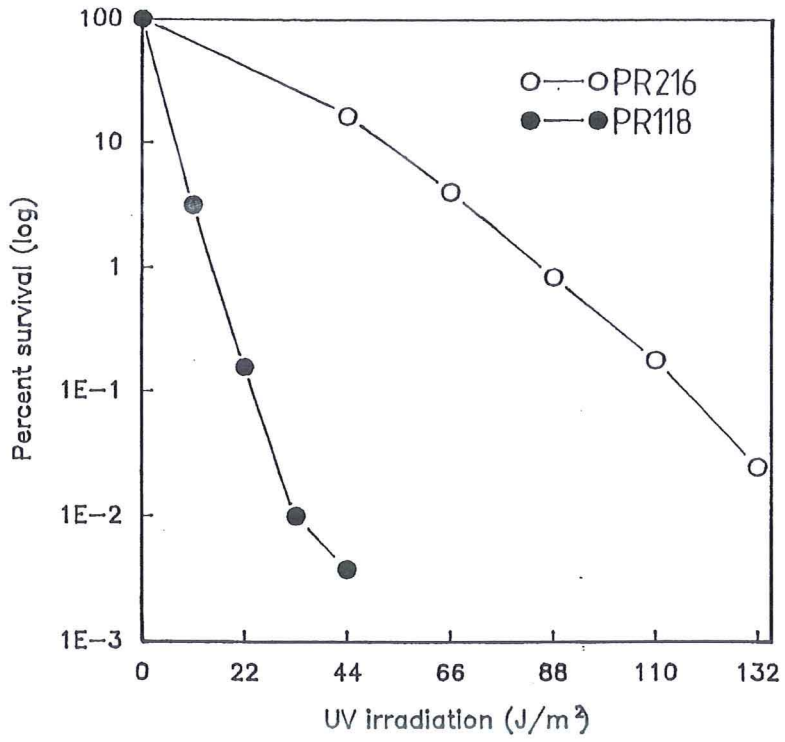


Fig. 5.2. Survival of PR216, a rifampicin resistant derivative of *P. putida* PMS118S and PR118, a potential recombination deficient derivative of PR216, after exposure to UV light (254 nm).

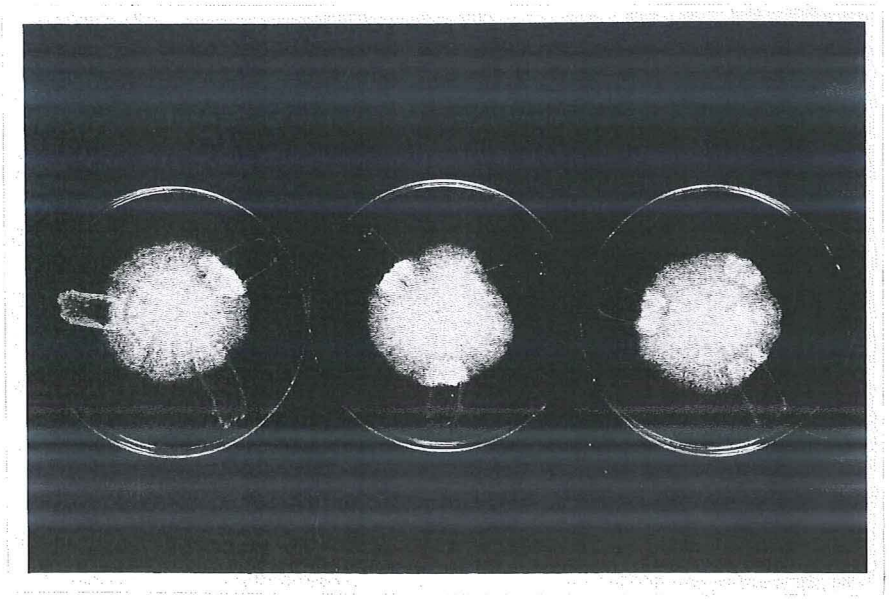


Fig. 5.3. The effect of a range of UV light generated, non-fluorescent mutants of *P. putida* PMS118S (produced at I.H.R.) on mycelial growth and basidiome initiation of *A. bitorquis* (W19).

the bacterial iron scavenging system in the process of basidiome initiation and further non-fluorescent, siderophore defective mutants were sought.

Table 5.3. Auxotrophic mutants of *P. putida* PMS118S

Mutant	Derivation	Method	Nutritional Requirement
PR116	PR118	Tn*5*	Arginine (Arg)
PR123	PR118	Tn*5*	Threonine (Thr)
PR124	PR118	Tn*5*	Tryptophan (Try)
PR125	PR118	Tn*5*	Tryptophan (Try)
PR126	PR118	Tn*5*	?: 3,4,6
PR127	PR118	Tn*5*	> 1 amino acid
PR129	PR118	EMS	?: 2,3,7,8
PR154	PR216	UV	Methionine (Met)
PR169	PR216	UV	Arginine (Arg)
PR176	PMS118	Tn*5*-Mob	Adenine
PR178	PMS118	Tn*5*-Mob	Methionine (Met)
PR179	PMS118	Tn*5*-Mob	Uracil
PR184	PR118	Tn*5*-Mob	Histidine (His)
PR185	PR118	Tn*5*-Mob	Histidine (His)
PR186	PR118	Tn*5*-Mob	?: 4
PR188	PR118	Tn*5*-Mob	Histidine (His)
PR192	PR216	Tn*5*	Tryptophan (Try)
PR193	PR216	Tn*5*	Threonine (Thr)
PR194	PR216	Tn*5*	Leucine (Leu)

? = nutritional requirements uncertain: numbers refer to nutritional pools of Davis *et al.* (1980) on which mutant grows

Table 5.4 lists the UV light generated siderophore defective mutants. Mutants unable to produce a fluorescent pigment on KB were screened against *A. bitorquis* W19 to determine their effect on basidiome initiation (Fig. 5.3). Not all non-fluorescent mutants inhibited basidiome initiation suggesting the involvement of a particular facet of the bacterial iron scavenging system. Further characterization of the mutants revealed that those unable to promote basidiome initiation (Bas$^-$) were, in addition to being defective in the biosynthesis and excretion of the yellow-green fluorescent siderophore and being unable to grow under iron limited conditions, also possessed a defective ferric-siderophore uptake system. This strongly implicated the membrane bound protein porins (responsible for uptake of the ferric-siderophore complex into the cell) in the process of basidiome initiation. More mutants were sought with similar defects and for ease of genetic analysis transposon mutagenesis was used.

Table 5.4. Siderophore defective mutants of *P. putida* generated by UV light and EMS

Mutant	Mutant class	Derivation	Method	Phenotype[1]	Basidiome initiation
PR144*	1	PMS118S	UV	$Flu^- Sid^+ lup^+$	+
PR120	2	PR118S	EMS	$Flu^- Sid^- lup^+$	+
PR121		PR118S	EMS	$Flu^- Sid^- lup^+$	+
PR145*		PMS118S	UV	$Flu^- Sid^- lup^+$	+
PR221*		PMS118R	UV	$Flu^- Sid^- lup^+$	+
PRS223*		PMS118R	UV	$Flu^- Sid^- lup^+$	+
PR224*		PMS118Rr	UV	$Flu^- Sid^- lup^+$	+
PR143*	3	PMS118S	UV	$Flu^- Sid^- lup^-$	-
PR222*		PMS118R	UV	$Flu^- Sid^- lup^{+/-}$	-
PR225*		PMS118R	SP[2]	$Flu^- Sid^- lup^-$	-
PR226*		PMS118R	UV	$Flu^- Sid^- lup^-$	-
PR119*	6	PR118S	EMS	$Flu^{+/-} Sid^{+/-} lup^+$	+
PR152*		PMS118S	UV	$Flu^{+/-} Sid^{+/-} lup^+$	+
PR153*		PMS118S	UV	$Flu^{+/-} Sid^{+/-} lup^+$	+
PR154-172		PR216	UV	Flu^{-3}	+
PR235-249		PR216	UV	Flu^-	+

[1] Flu^-, no fluorescence; $Flu^{+/-}$, moderate fluorescence; Flu^+, wild type fluorescence; Sid^- no growth in the presence of EDDA; $Sid^{+/-}$, growth in presence of 0.31, 0.62, or 1.25 mg ml^{-1} EDDA; Sid^+, normal (wild type) growth in the presence of 5.0 mg ml^{-1} EDDA; lup^-, unable to utilize wild type siderophore; lup^+, able to utilize wild type siderophore. Fluoresence was determined by irradiation with UV light.

[2] Spontaneously derived mutant

[3] These mutants were not characterized further

* Produced at I.H.R.

Transposon Tn5 mutagenesis of *P. putida* PMS118S was conducted initially at I.H.R. Following mating of donor strain *E. coli* S17-1, containing pSUP2021, with recipient strain PMS118S, kanamycin and naladixic acid resistant transconjugants were found with a frequency of 3.0×10^{-6} per recipient cell. Fifty transconjugant colonies were chosen at random and their sensitivity to tetracycline determined; all remained sensitive indicating that the suicide plasmid had been lost. Loss of the suicide plasmid was further confirmed by screening five transconjugant colonies for the presence of plasmid DNA; none contained an additional plasmid band. Auxotrophic mutants were found among the kanamycin and naladixic acid transconjugants with a frequency of 1 % indicating that integration of the transposon into the PMS118S genome occurred randomly (Marugg *et al.* 1985).

At I.H.R. 500 kanamycin resistant PMS118S colonies were screened for mutants defective in the biosynthesis (or excretion) of the yellow-green fluorescent siderophore. Mutants with this phenotype were screened against W19 to determine their effect on basidiome initiation. As found previously, not all non-fluorescent colonies inhibited basidiome initiation. Nine siderophore defective cells were found and are listed in Table 5.5. Only one of these failed to promote

Table 5.5. Siderophore defective mutants of *P. putida* generated by transposon Tn*5* mutagenesis

Mutant	Mutant class	Derivation	Phenotype[1]	Basidiome initiation
PR109	1	PR118S	$Flu^- Sid^+ Iup^+$	+
PR132		PR118S	$Flu^- Sid^+ Iup^+$	+
PR148*		PMS118S	$Flu^- Sid^+ Iup^+$	+
PR101	2	PR118S	$Flu^- Sid^- Iup^+$	+
PR112		PR118S	$Flu^- Sid^- Iup^+$	+
PR127		PR118S	$Flu^- Sid^- Iup^+$ (Aux)[2]	+
PR137		PR118S	$Flu^- Sid^- Iup^+$	+
PR138		PR118S	$Flu^- Sid^- Iup^+$	+
PR139		PR118S	$Flu^- Sid^- Iup^+$	+
PR146*		PMS118S	$Flu^- Sid^- Iup^+$	+
PR147*		PMS118S	$Flu^- Sid^- Iup^+$	+
PR149*		PMS118S	$Flu^- Sid^- Iup^+$	+
PR150*	3	PMS118S	$Flu^- Sid^- Iup^-$	-
PR106	4	PR118S	$Flu^+ Sid^+ Iup^-$	+/-
PR110		PR118S	$Flu^+ Sid^+ Iup^-$	+/-
PR126		PR118S	$Flu^+ Sid^+ Iup^-$ (Aux)	+/-
PR111	5	PR118S	$Flu^{+/-} Sid^- Iup^+$	+
PR105	6	PR118S	$Flu^{+/-} Sid^{+/-} Iup^+$	+
PR151		PMS118S	$Flu^{+/-} Sid^{+/-} Iup^+$	+
PR227*		PMS118S	$Flu^{+/-} Sid^{+/-} Iup^+$	+
PR228*		PMS118S	$Flu^{+/-} Sid^{+/-} Iup^+$	+
PR113	7	PR118S	$Flu^{+/-} Sid^+ Iup^+$	+
PR115		PR118S	$Flu^{+/-} Sid^+ Iup^+$	+
PR125		PR118S	$Flu^{+/-} Sid^+ Iup^+$ (Aux)	+
PR131		PR118S	$Flu^{+/-} Sid^+ Iup^+$	+
PR133		PR118S	$Flu^{+/-} Sid^+ Iup^+$	+
PR229*		PMS118S	$Flu^{+/-} Sid^{+/-} Iup^+$	+
PR230*	8	PMS118S	$Flu^+ Sid^{+/-} Iup^+$	+

[1] See bottom of Table 5.4 for explanation of abbreviations

[2] Auxotroph - see Table 5.3.

* Produced at I.H.R.

basidiome initiation, PR150, and this possessed the same phenotype as the UV generated basidiome inhibitory (Bas$^-$) mutants, that is, in addition to being non-fluorescent and non-siderophore producing, it was also unable to utilize the siderophore produced by the parent strain.

At Canterbury University more Tn*5* generated, siderophore defective mutants were produced, however, despite screening more than 2000 independently generated Tn*5* mutants, none were found which inhibited basidiome initiation as markedly as the mutants produced at I.H.R., and none possessed a phenotype identical to PR150. Siderophore-iron uptake mutants were found; PR106, PR110 and PR126, but, unlike the I.H.R. Bas$^-$ mutants, these produced and excreted a functional siderophore (Table 5.5 and see below). The Canterbury University ferric-siderophore uptake mutants did not promote basidiome initiation to the same extent as the wild type PMS118S, but their ability to inhibit fruiting was variable (Fig. 5.4).

It was quite possible that more than a single gene was inactivated in the Bas$^-$ UV generated mutants, especially as UV light induces deletion mutations. Consequently, further deletion mutants were sought. It was desirable to generate these mutants in a Rec$^-$ background (for subsequent gene cloning work), but, because of the extreme sensitivity of PR118 to UV light (all Rec$^-$ strains possess this trait) it was not possible to mutate this strain using UV light. Instead, an attempt was made to use nitrous acid, another deletion inducing mutagen. Nitrous acid, however, proved unsuitable for generating mutants and irrespective of recombinational proficiency, nitrous acid killed all *P. putida* cells which it contacted. This occurred even when the concentration of nitrous acid was reduced one hundred fold (0.5 mM) and the cells exposed to it for only 30 s.

UV light, which had worked successfully at I.H.R., was once again used to generate basidiome inhibitory mutants. The UV light source at Canterbury University was calibrated and Fig. 5.2 shows the survival of *P. putida* strains PMS118S/PR216 after exposure to UV light. The rifampicin resistant derivative of PMS118S, PR216, was mutagenized, but despite generating more than 30 non-fluorescent mutants, none were found which inhibited basidiome initiation.

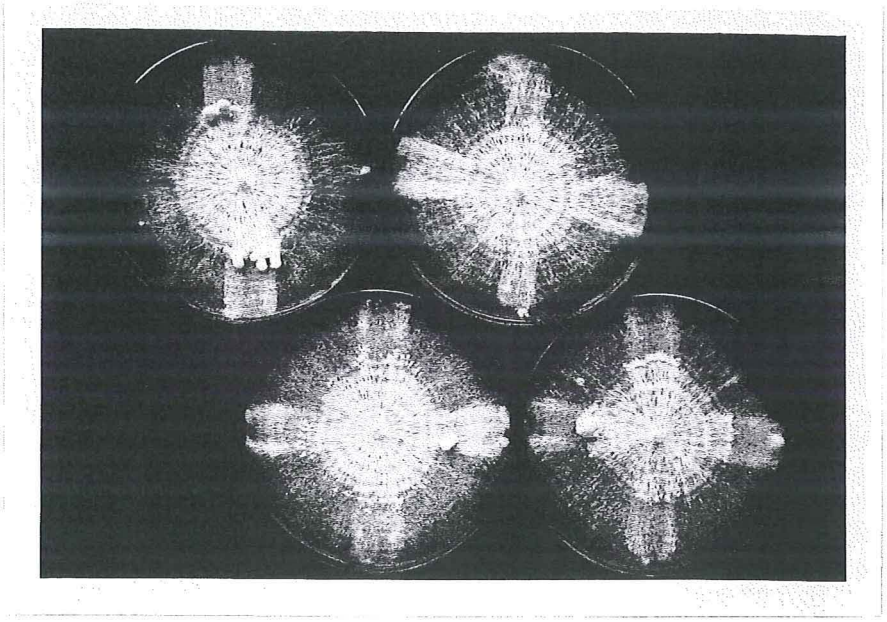


Fig. 5.4. The variable effect of a range of Tn*5* generated mutants of *P. putida* PR118 (produced at Canterbury University) on basidiome initiation of *A. bitorquis* W19. Top LH Petri dish; PR118 (control) top and bottom, PR190 left and right. Top RH Petri dish; PR201 top and bottom, PR200 left and right. Bottom LH Petri dish; PR198 top and bottom, PR199 left and right. Bottom RH Petri dish; PR106 top and bottom, PR110 left and right. PR110 and PR106 are Flu^+ Sid^+, lup^-; PR190, PR198, PR199 and PR200 were selected after screening directly against W19 for basidiome inhibitory mutants (see text).

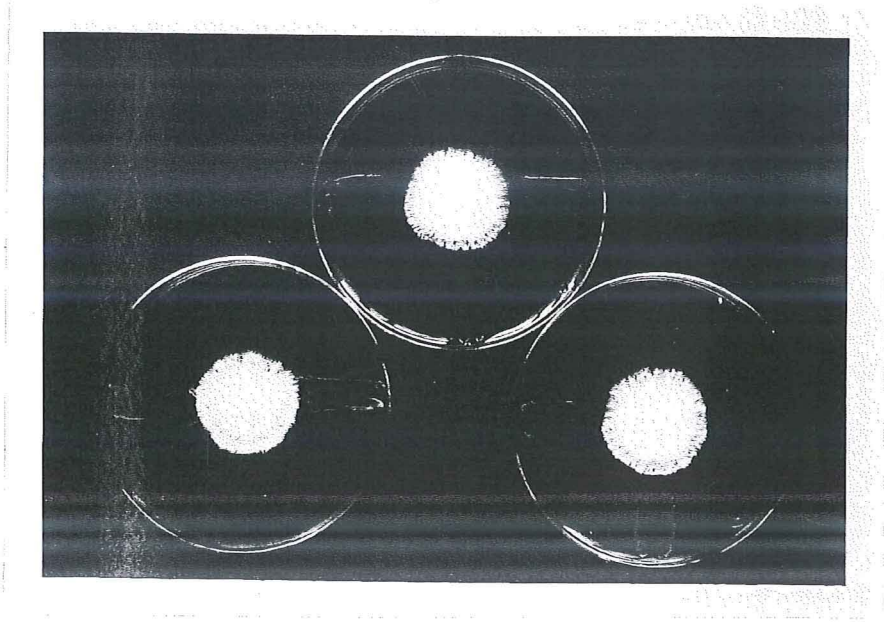


Fig. 5.5. The effect of *A. bitorquis* W19 on the growth of Tn*5* generated mutants of *P. putida* PR118 defective in their ability to promote basidiome initiation. Bottom LH Petri dish; PR118 (control) left and right, PR190 top and bottom. Top Petri dish; PR199 left and right, PR198 top and bottom; Bottom RH Petri dish; PR200 left and right, PR201 top and bottom. Bacteria have been growing in the presence of the fungus for less than 24 h. Note the inhibition of bacterial growth in all instances except the control.

The frequency of non-fluorescent mutants was much less than was observed at I.H.R.; ca. 1.0 %, compared with ca. 2-3 %.

Having been unable to generate non-fluorescent basidiome inhibitory mutants at Canterbury University, it was decided that Tn5 mutants should be generated and screened directly for their inability to promote basidiome initiation, using the *A. bitorquis* W19 bioassay. PR118 was subjected to transposon Tn*5* mutagenesis using both pSUP2021 and pSUP5011 (only 100 pSUP5011 containing colonies were screened against W19 as it was possible that the presence of the Mob gene, which is derived from RP4, may have been incompatible with the RP4::mini-Mu cloning system). Eight hundred independently generated kanamycin resistant PR118 colonies were screened against W19 and four colonies were found which failed to promote fruiting. All grew poorly on CMM in the presence of W19 (Fig. 5.5), but grew normally on this substrate when the fungus was absent. PR190 was particularly unusual; it grew well on KB and produced a fluorescent pigment, however, after 36 - 48 h growth, the fluorescent pigment disappeared. In addition, PR190 was oxidase negative after growth on CMM, but oxidase positive after growth on KB. Fig. 5.4 shows the effect of the basidiome inhibitory mutants, PR190, PR200, PR201 and PR198 on basidiome initiation.

Antibiotic resistance of Bas⁻, siderophore defective mutants

Table 5.6 shows the antibiotic resistance patterns of the Bas⁻, siderophore defective mutants. Only one mutant, PR222, showed the same antibiotic resistance pattern of the wild type.

Table 5.6. Antibiotic resistance of basidiome inhibitory, iron uptake *P. putida* mutants

Mutant	**Antibiotic**						
	Am	**Cm**	**Km**	**Nx**	**Sm**	**Tc**	**Em**
PMS118S	R[1]	R	S	R	S	S	R
PR143	S	S	S	S	S	S	S
PR150	R	ND	ND	ND	S	S	S
PR222	R	R	S	R	S	S	R
PR225	R	S	R	S	S	S	R
PR226	R	S	R	R	S	S	R

[1]R, resistant; S, sensitive; ND, not determined

Siderophore defective mutants

Eight different classes of siderophore defective mutants were found. The first group consisted of mutants which did not produce a fluorescent yellow-green pigment on KB, but which grew under iron limitation (KB plus 5.0 mg ml^{-1} EDDA). Mutants of class 2 did not produce a fluorescent pigment, nor were they able to grow under iron limited conditions. They were, however, able to grow under conditions of iron limitation if the wild type siderophore was present in the media. Class 3 mutants were identical to class 2 mutants, but in addition, they were unable to utilize the siderophore produced by the parent strain. Class 4 mutants were unusual in that they produced a functional, yellow-green fluorescent siderophore (Determined by checking fluorescence on KB and by growing the mutants in KB broth, removing the cells by filtration and placing a drop alongside class 2 mutants which had been plated on iron limited media. The siderophore produced by these mutants enabled growth of the Sid^-, class 2, mutants.), but were unable to take up the ferric-siderophore complex. Consequently they grew slowly on media such as KB and sometimes a yellow-green fluorescent pigment was produced on iron replete media, such as nutrient agar. Another interesting feature of these mutants was that they lacked cyctochrome *c* (as determined by the oxidase reaction (Kovac 1956)). They also tended to be unstable. The class 5 mutant was moderately fluorescent, but was unable to grow under iron limited conditions. Class 6 mutants possessed an intermediate phenotype and were slightly affected in both fluorescence and growth under iron limited conditions. Mutants of class 7 produced a yellow-green fluorescent pigment which was clearly less intense than the wild type, but which grew in the presence of 5.0 mg ml^{-1} EDDA. The class 8 mutant showed fluorescence like the wild type, but was affected in its ability to reverse iron limitation induced by EDDA.

5.3.2. DEVELOPMENT OF AN *IN VIVO* GENE CLONING AND MAPPING SYSTEM (RP4::MINI-MU) IN *P. PUTIDA*

The ability of basidiome stimulatory *P. putida* isolates to accept and donate RP4 was examined before beginning work with the RP4::mini-Mu *in vivo* cloning system. RP4 was transferred efficiently into two of four wild type *P. putida* strains and revealed the suitability of these isolates for genetic analysis using gene cloning systems based on RP4 derived plasmids. RP4 was

compatible with the two unmarked cryptic plasmids harboured by *P. putida* PMS118S and was transferred efficiently from *P. putida* PMS118S and PMS196, in both homologous and heterologous matings. Isolates PMS118S and PMS196 were considered good candidates for *in vivo* gene cloning (Table 5.7, Fig. 5.6).

Table 5.7. Transfer of RP4 amongst *P. putida* strains

Donor		**Recipient**		**Plasmid transfer frequency**[1]
E. coli	HB101	*P. putida*	PMS118S	4×10^{-1}
			PMS195	$< 10^{-8}$
			PMS196	2×10^{-2}
			PMS234	$< 10^{-8}$
P. putida	PMS118S	*P. putida*	PR220	7×10^{-2}
		E. coli	HB101	1×10^{-1}
		P. aeruginosa	PAO2003	5×10^{-1}
			OT11	8×10^{-1}
		S. entomophila	IS1	3×10^{-3}
	PMS196	*P. putida*	PR216	4×10^{-2}
		E. coli	HB101	9×10^{-2}
		P. aeruginosa	PAO2003	5×10^{-2}
			OT11	9×10^{-4}
P. aeruginosa	OT899	*E. coli*	HB101	5×10^{-1}

[1]Estimated per recipient cell.
E. coli HB101 donors were selected against with Nx; *P. putida* donors were selected against with Rm when the recpient was *P. putida* and Sm when the recipient was *E. coli* or *P. aeruginosa*; *P. aeruginosa* donors were selected against with Sm

Difficulties were encountered with the RP4::mini-Mu system which arose from instability of the mini-Mu containing plasmids and limited the progress of this work. Plasmid pULB113 was the plasmid of choice for *in vivo* cloning in *P. putida* PMS118S, because of its antibiotic resistance markers. The initial *E. coli* strain containing pULB113, obtained from the Unit of Nitrogen Fixation, University of Sussex, U.K., was shown to possess the correct antibiotic markers and strains carrying the plasmid were lysed by Mu*cts*62 at 42 °C but not at 30 °C, however, pULB113 would not transfer in homologous or heterologous matings. Restriction endonuclease digestion of cells containing the plasmid revealed deletions from the two largest *Pst*I fragments on which are located the RP4 transfer genes (Fig. 5.7). Analysis of a second pULB113 containing strain

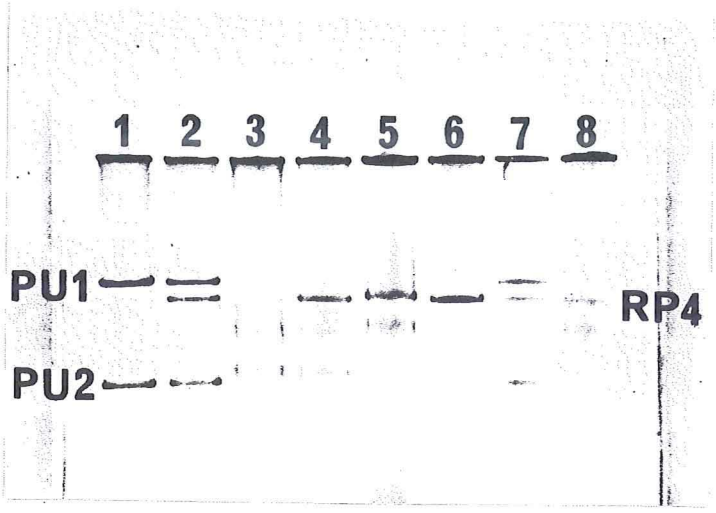


Fig. 5.6. Electropherogram of plasmid DNA extracted from *P. putida* strains believed to have aquired the broad host range plasmid RP4. Lane 1, *P. putida* PMS118S showing the cryptic plasmids pPU1 (ca. 80 kb, see Appendix C) and pPU2 (ca. 24 kb, see Appendix C); lanes 2 and 7, *P. putida* PMS118S containing RP4; lane 3, *P. putida* PMS196 (plasmidless strain); lanes 4 and 8, *P. putida* PMS196 containing RP4; lane 5, *E. coli* HB101 (donor strain) containing RP4.

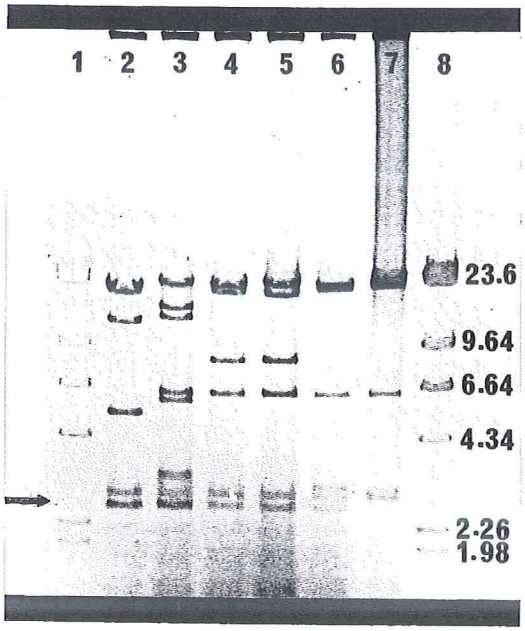


Fig. 5.7. Electropherogram of *Pst*I digested RP4::mini-Mu plasmids. Lanes 1 and 8 contain lambda *Hin*dIII markers (kb); lane 2 contains pULB113 (University of Sussex); lane 3 contains pULB113 (University of Otago); lanes 4 and 5 contain pULB21 (University of Sussex); lane 6 contains pULB110 (University of Sussex); lane 7 contains RP4. Arrow shows internal mini-Mu fragment (2.9 kb). (Physical maps of the RP4::mini-Mu plasmids are given in Fig. 5.7a).

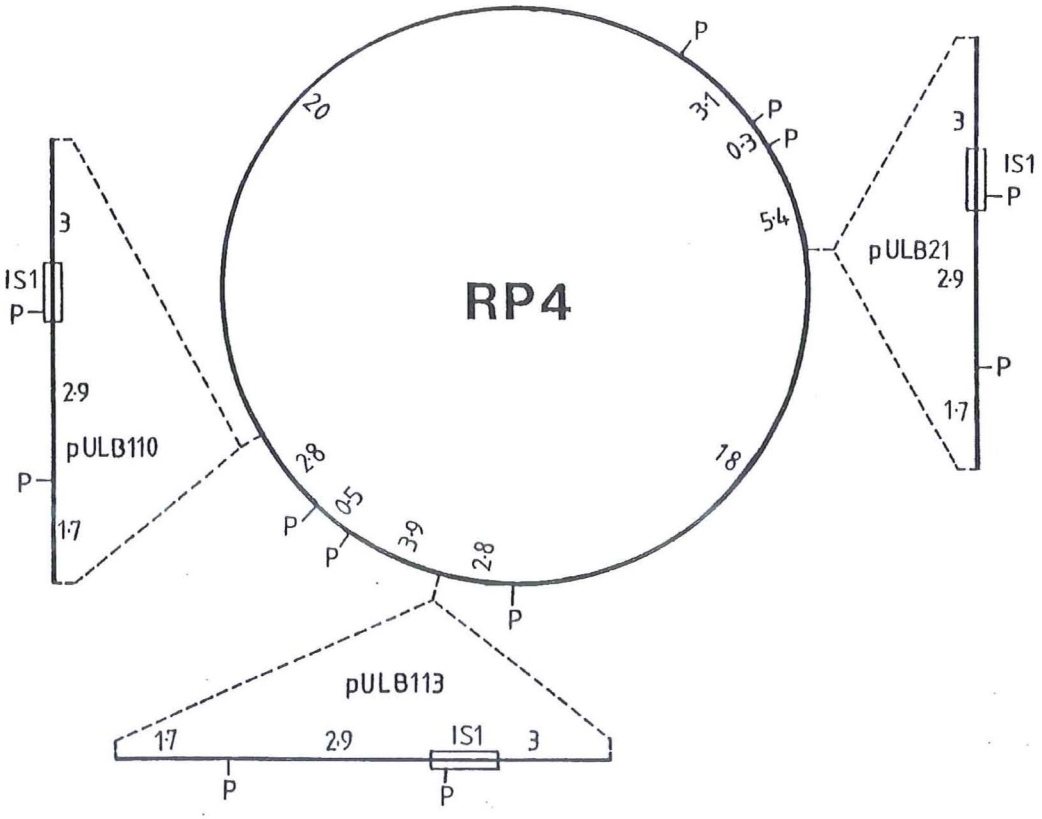


Fig. 5.7a. Physical maps of the three RP4::mini-Mu plasmids, pULB113, pULB21 and pULB110 (from Van Gijsegem & Toussaint 1982). P, *Pst*I sites; distances are in kb.

obtained from Dept. of Microbiology, Otago University, revealed similar defects (Fig. 5.7). Plasmids pULB21 and pULB110 both transferred well in homologous and heterologous matings, possessed the correct antibiotic markers and were immune to Muc*ts*62 at 30 °C. Restriction endonuclease digestion revealed pULB21 to be physically intact, however, pULB110 was missing a 4.5 kb fragment which encompasses one of the ends of the mini-Mu fragment and is necessary for expression of Mu encoded traits, such as transposition (Fig. 5.7).

A fresh culture of pULB113 was obtained from Van Gijsegem & Toussaint, Laboratoire de Genetique, University Libre de Bruxelles, Belgium, and plated on antibiotic containing media. Mixed inoculum was removed from the selective plate and mated with *P. putida* PMS118S and kanamycin, tetracycline and naladixic acid resistant transconjugants were found at a high frequency (ca. 10^{-3} per recipient cell) indicating transfer of the plasmid to the *P. putida* strain. Six individual *E. coli* colonies, plus mixed inoculum were then selected from the antibiotic amended plate and examined for expression of Mu immunity and plasmid integrity. All six, plus the 'mixture', were immune to Muc*ts*62 at 30 °C, but only a small number of plaques were visible in the 'mixture' indicating that not all cells expressed Mu immunity. The 'mixture' revealed the correct pattern of bands following digestion with *Pst*I (Fig. 5.8), however, a number of faint additional bands were also present indicating that many cells contained altered plasmid DNA. Three of the six individual colonies displayed atypical banding patterns and transposition of chromosomal DNA onto pULB113 (R-prime formation) appeared to have already occurred (Fig. 5.8). The other three colonies appeared to give the correct pattern of bands and were stored in glycerol, at -80 °C to be used as donors in subsequent matings. However, these plasmids did not transfer to either *E. coli* HB101 or *P. putida* PMS118S and possibly contained deletions from the large *Pst*I fragments on which the transfer functions are located. Detection of these deletions is difficult, because the distance migrated by the large fragments is not greatly affected by loss of a small fragment of DNA.

Further single *E. coli* MXR(pULB113) colonies were selected from antibiotic amended plates and screened individually for expression of Mu immunity and intact transfer functions (by mating with *E. coli* HB101). Cells possessing these traits were then subjected to restriction

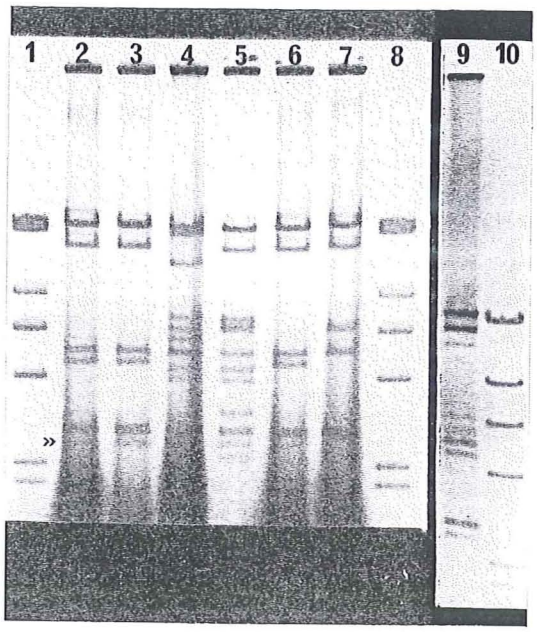


Fig. 5.8. Electropherogram of pULB113 DNA extracted from single *E. coli* MXR colonies (obtained from Universite Libre de Bruxelles) and digested with *Pst*I. Lanes 2, 3 and 6 contain what was believed to be physically intact pULB113 - this was later shown to be incorrect (see Fig. 5.9 and text). The plasmids contained in lanes 4 and 5 have aquired additional DNA inserts typical of R-prime plasmids. Lane 9 contains pULB113 DNA prepared from a mixture of colonies. The bright bands show the correct pattern of bands for pULB113 (see Fig. 5.7a), but the presence of additional faint bands indicates that a large number of plasmids contain additional DNA. Lanes 1, 8 and 10 contain lambda standards as in Fig. 5.7. Arrow shows internal mini-Mu fragment (2.9 kb).

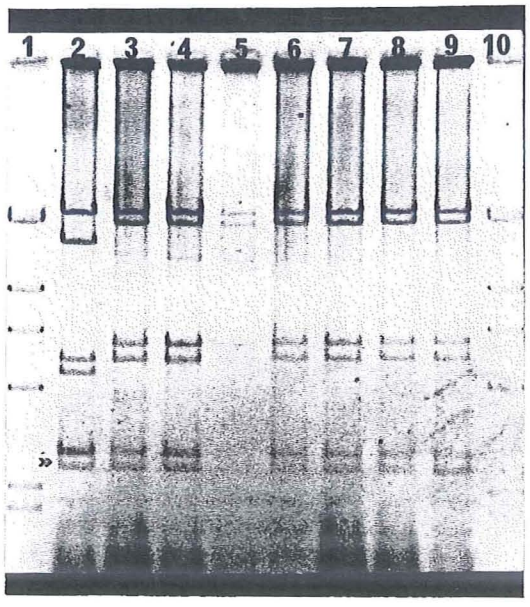


Fig. 5.9. Electropherogram of pULB113 DNA extracted from single *E. coli* MXR colonies (obtained from Universite Libre de Bruxelles) and digested with *Pst*I. The plasmids in lanes 3 - 9 had been shown to transfer in homologous matings and confer immunity to Mu at 30 °C. Lane 2 contains plasmid DNA isolated from a colony which expressed Mu immunity and the correct antibiotic markers, but which failed to transfer in either homologous or heterologous matings. A deletion can be seen on the second largest fragment. Lanes 1 and 10 contain lambda standards as in Fig. 5.7. Arrow shows interanl mini-Mu fragment (2.9 kb).

endonuclease digestion. Less than 50 % of the colonies tested possessed intact transfer functions, but those which did, also showed the correct pattern of bands following *Pst*I digestion. Fig. 5.9 shows these plasmids and also shows the banding pattern of one of the plasmids previously thought to be physically correct; a deletion from the second to largest band is clearly visible when this plasmid was digested alongside a physically intact pULB113 plasmid. This deletion would have accounted for its inability to conjugate in previous matings. Several of the colonies containing intact plasmids were stored in glycerol for use in subsequent matings.

Physically intact RP4::mini-Mu plasmids transferred efficiently into *P. putida* strains PMS118S and PMS196 and were stably maintained. This was tested by looking for the presence of pULB113 markers after several subcultures in non-selective media and testing for expression of Mu immunity after transfer of the plasmid back into *E. coli* HB101. Table 5.8 shows the results of homologous and heterologous matings made with these strains and with *E. coli* and *P. aeruginosa*.

Homologous matings

In *E. coli* MXR, pULB113 mediated transfer of leucine; proline was not transferred as the donor strain is a proline auxotroph. In *P. putida* PMS118S, pULB113 mediated transfer of methionine and arginine genes and in *P. putida* PMS196, pULB113 mediated transfer of the gene(s) required to complement an arginine auxotroph. Unfortunately, no multiple auxotrophs of *P. putida* strains were made and therefore it was not possible to determine transfer of unselected markers indicative of chromosome mobilization, however, analysis of plasmid DNA from *P. putida* recipients revealed the absence of additional DNA inserts which suggests that complementation occurred as the result of chromosome mobilization. Complementation of *P. putida* PR118 (Rec^-) Tn*5* generated auxotrophs was unsuccessful. This was possibly caused by the presence of transposon Tn*5* within the *P. putida* genome which may have been incompatible with the RP4::mini-Mu plasmid.

Table 5.8. Transfer of pULB113, pULB21 and pULB110 and transfer of chromosomal markers mediated by pULB113

Donor		Recipient		Plasmid transfer frequency[1]	Selected marker	Marker transfer frequency[1]
E. coli	MXR(pULB113)	*P. putida*	PMS118S	8×10^{-2}		
			PMS196	6×10^{-3}		
		P. tolaasii	PMS117S	4×10^{-4}		
		P. aeruginosa	PAO2003	5×10^{-2}		
			OT11	8×10^{-3}		
		E. coli	HB101	7×10^{-2}	Leu	4.2×10^{-6}
					Pro	0
			RR1	5×10^{-2}	Leu	3×10^{-5}
					Pro	0
E. coli	MXR(pULB21)	*P. putida*	PMS118S	5×10^{-2}		
			PMS196	5×10^{-2}		
		P. tolaasii	PMS117S	6×10^{-4}		
		P. aeruginosa	OT11	6×10^{-3}		
		E. coli	HB101	9×10^{-2}		
E. coli	MXR(pULB110)	*P. putida*	PMS118S	7×10^{-2}		
			PMS196	3×10^{-2}		
P. putida	PMS118S(pULB113)	*E. coli*	HB101	5×10^{-2}	Leu	1.2×10^{-6}
					Pro	$< 10^{-8}$
			RR1	8×10^{-2}	Leu	1.0×10^{-5}
					Pro	1.0×10^{-5}
		P. putida	PR123	3×10^{-3}	Thr	$< 10^{-8}$
			PR154	8×10^{-3}	Met	7×10^{-7}
			PR169	5×10^{-2}	Arg	1×10^{-5}
			PR185	4×10^{-2}	His	$< 10^{-8}$
			PR194	8×10^{-3}	Leu	$< 10^{-8}$
		P. aeruginosa	PAO2003	6.0×10^{-3}	Arg	$< 10^{-8}$
			OT11	1×10^{-3}	Leu	1×10^{-7}
					Pro	$< 10^{-8}$
P. putida	PMS196(pULB113)	*E. coli*	HB101	8×10^{-2}	Leu	5×10^{-7}
					Pro	$< 10^{-8}$
		P. putida	PR169	4×10^{-2}	Arg	5×10^{-6}

[1] Estimated per recipient cell.

E. coli donors were selected against with Nx, Sm was used when the recpient was *E. coli*. *P. putida* donors were selected against with Sm, Rm was used when the recipient was *P. putida*.

Spontaneous reversion of auxotrophs $< 5 \times 10^{-8}$

Heterologous matings

In *P. putida* PMS118S, pULB113 mediated transfer of leucine and proline to *E. coli* RR1, but only leucine to *E. coli* HB101. Plasmid pULB113 in *P. putida* PMS118S also mediated transfer of leucine to *P. aeruginosa* OT11. In *P. putida* PMS196, pULB113 mediated transfer of leucine to *E. coli* HB101, but not proline. No recombinants inherited unselected markers indicating that R-prime plasmids, and not chromosome mobilization, was most likely responsible for complementation of the mutations.

Plasmid DNA was isolated from *E. coli* strains, HB101 and RR1, complemented for either leucine or proline by *P. putida* PMS118S genes transferred on pULB113, but while all possessed a plasmid band corresponding to pULB113, none showed additional inserts. This was surprising as R-prime formation would be expected in heterologous matings, especially those between *P. putida* and the enteric bacterium, *E. coli*, regardless of the recombinational status of the recipient. It was possible that R-prime plasmids containing *P. putida* genes were unstable in *E. coli* and lost during overnight growth in L-broth, prior to plasmid DNA extraction. To test this, *E. coli* HB101 cells were grown on solid M9 medium (growth in M9 broth was extremely poor) and after 3 d cells were harvested and the plasmid DNA prepared. Two out of six colonies possessed plasmid DNA with additional inserts (Fig. 5.10). One one of these colonies showed two bands; one of which resembled pULB113, while the other was considerably larger. This large (R-prime) plasmid band was also fainter and was probably in the process of loosing its *P. putida* derived DNA.

5.3.3. MARKING AND MOBILIZATION OF A CRYPTIC *P. PUTIDA* PMS118S PLASMID

Attempts to transfer the two *P. putida* PMS118S plasmids; pPU1 and pPU2, by conventional methods had proved unsuccessful, therefore, the Tn*5*-Mob system (Simon 1984) for marking and mobilizing cryptic plasmids was investigated. Transposon Tn*5*-Mob was introduced into *P. putida* PMS118S form the *E. coli* donor strain S17-1. Kanamycin and naladixic acid resistant transconjugants were obtained at a frequency of 7.5×10^{-6} per recipient cell. A total of 30 kanamycin resistant clones were mated individually with *E. coli* containing pJB3JI, and

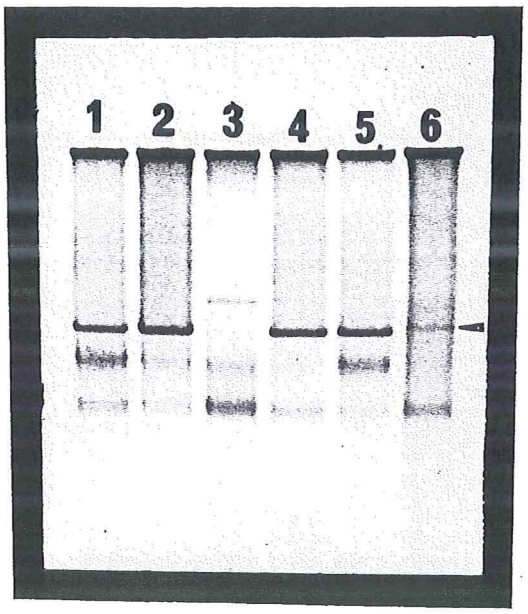


Fig. 5.10. Electropherogram of pULB113 DNA extracted from *E. coli* HB101 colonies complemented with the leucine gene from *P. putida* PMS118S. DNA was prepared from 3 d colonies scraped off minimal M9 medium (supplemented with proline) (see text). The plasmid (R-prime) in lane 3 contains a large additional fragment of DNA. Lane 6 also shows a plasmid with additional DNA (faint band) and a second brighter band corresponding to pULB113 (arrow). The R-prime in lane 6 is probably in the process of losing the *P. putida* derived DNA. The plasmids in lanes 1, 2, 4 and 5 appear to have already lost their *P. putida* derived DNA.

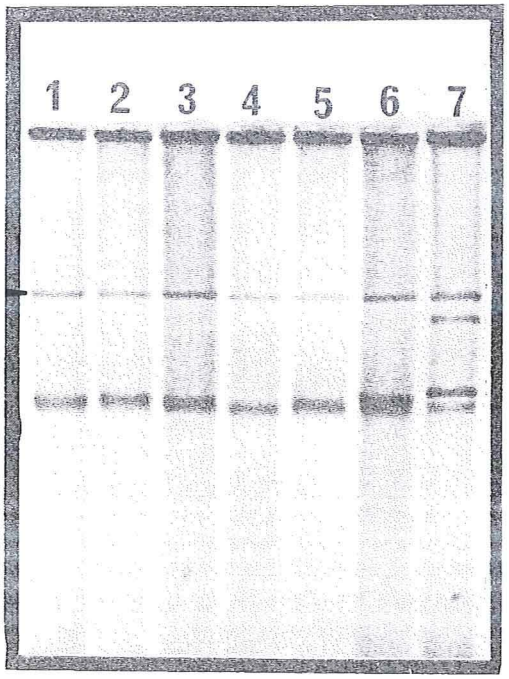


Fig. 5.11. Electropherogram of plasmid DNA extracted from *P. putida* PR220 colonies believed to have aquired a cryptic *P. putida* PMS118S plasmid containing Tn*5*-Mob. Lanes 1 - 6 show pPU1 DNA (arrow) isolated from six single PR220 colonies. Lane 7 shows pPU1 (largest band), pPU2 and the helper plasmid pJB3JI (middle band) extracted from *P. putida* PMS118S.

tetracycline and kanamycin resistant transconjugants were obtained at a high frequency (ca. 10^{-1} per recipient cell). One clone from each mating was selected, purified and mated with *E. coli* HB101. No streptomycin, kanamycin resistant transconjugants were obtained from this mating indicating that neither of the cryptic plasmids had been transferred to HB101. It was possible that Tn*5*-Mob may not have inserted into one of the PMS118S plasmids, but also possible that the transposon did insert, but because of its narrow host range was unable to replicate in *E. coli*.

A second experiment was conducted (as described in section 5.2.5.). After selecting 100 kanamycin and naladixic acid resistant PMS118S cells and mating these *en masse* with *E. coli* containing pJB3JI followed by an *en masse* mating with PR220 (a rifampicin resistant derivative of *P. putida* PMS196), rifampicin and kanamycin resistant transconjugants were found at a frequency of 4.1×10^{-4} per recipient cell. The presence of a plasmid of approximately 80 kb, corresponding to pPU1, was detected in the *P. putida* PR220 recipient (Fig. 5.11).

To investigate the host range of pPU1, the helper plasmid pJB3JI was introduced into PR220 and pPU1 mobilized into *E. coli* HB101 and *S. entomophila* IS1. The PMS118S plasmid pPU1 transferred into these strains at a frequency of 1.0×10^{-3} and 2.6×10^{-4}, respectively. Three clones of HB101(pPU1) and IS1(pPU1) were selected, the plasmid DNA prepared from each and run out on an agarose gel. A plasmid corresponding to pPU1 was found in all three *S. entomophila* clones, but in only one of the *E. coli* clones. Neither HB101(pPU1), nor IS1(pPU1) were able to promote basidiome initiation of W19.

5.4. DISCUSSION

5.4.1. MUTANTS OF *P. PUTIDA* PMS118S ALTERED IN THEIR ABILITY TO PROMOTE BASIDIOME INITIATION OF *A. BISPORUS*

Isolation of non-fluorescent *P. putida* PMS118S mutants which inhibited basidiome initiation of *A. bitorquis* W19 and failed to promote the rate of mycelial growth of *A. bisporus*, implicated the bacterial iron scavenging system in the process of basidiome initiation. The direct involvement of both iron and the *P. putida* PMS118S siderophore in fruiting of *A. bisporus* was examined and discussed in Chapter 4.0. (see section 4.4.3.) and a role for these compounds was discounted.

Further characterization of the basidiome inhibitory mutants revealed that in addition to being non-fluorescent, they were also unable to produce a functional siderophore, and lacked the ability to import the ferric-siderophore complex. Mutants which were defective in siderophore biosynthesis or excretion (Flu^- and/or Sid^-), but possessed a functional uptake system, failed to inhibit basidiome initiation, while mutants which were solely iron uptake defective, only partially inhibited fruiting. This result implicated the ferric-siderophore uptake system in the fruiting process and the inactivation of both siderophore biosynthesis (and or excretion) and the ferric-siderophore uptake system indicates the involvement of several bacterial genes. The failure of Flu^+, Sid^+, Iup^-, mutants to fully suppress basidiome initiation suggests that siderophores may play some part in the process, although this is difficult to explain, as siderophore production on CMM is suppressed (see 4.4.3.). It is possible that the *P. putida* siderophore is not directly involved in the fruiting response, but is affected indirectly by an uncharacterized mutation.

The ferric-siderophore uptake system and its possible involvement in basidiome initiation

The ferric-siderophore uptake system consists of a range of membrane bound porin proteins and cytoplasmic bound proteins which display a high affinity for the ferric-siderophore complex (Neilands 1982). These proteins are induced under iron limiting conditions, but are present in low numbers even when growth occurs under iron replete conditions (Hider 1984). Removal of ferric iron from the ferric-siderophore complex is also part of the uptake process and is thought to occur either directly after binding of the complex to the receptor, or after transport of the ferric-

siderophore complex into the cell (Neilands 1982). Outer membrane proteins have been shown to be involved in iron uptake by a variety of micro-organisms, including *Salmonella typhimurium* (Bennett & Rothfield 1976), *E. coli* (Hollifield & Neilands 1978), *P. aeruginosa* (Sokol & Woods 1983) and *P. putida* (de Weger *et al.* 1986, Magazin *et al.* 1986). The protein receptors are highly specific for the ferric-siderophore, however, in *E. coli* they have also been shown to enable bacteriophages, bacteriocins and some high molecular weight nutrient compounds, such as vitamin B_{12}, to enter the cell (see Neilands 1982 and Osborn & Wu 1980).

It is possible that the membrane bound transport proteins are involved in uptake of the 'self-inhibitory-compound' thought to be produced by *A. bisporus* mycelium (Eger 1961, Wood 1976, see Chapter 4.0.). A defect in these protein porins may prevent uptake and metabolism of the 'self-inhibitory-compound' which would result in inhibition of basidiome initiation. Membrane bound proteins also enable excretion of compounds and it is therefore possible that these proteins may enable excretion of a 'basidiome stimulatory compound', but as discussed previously (see section 4.4.3.), the production of such a compound by *P. putida* does not seem likely.

The failure of the ferric-siderophore uptake, basidiome inhibitory mutants to give a positive oxidase reaction indicates the absence of cytochrome *c*. Whether the absence of cytochrome *c* was a direct result of the mutation, or was caused indirectly by the shortage of iron (iron forms the centre of the heme group which is the central part of cytochrome *c* (Dickerson 1980)) is not known. Nevertheless, this discovery suggests an energy requiring stage in the process of basidiome initiation. Energy is necessary for transport of the ferric-siderophore complex into the cell, and for removal and reduction of ferric iron from the siderophore (Neilands 1982, Hider 1984), and if the proposed 'self-inhibitory-compound' is taken into the cell via the protein porins, then it is likely that degradation of it would also be dependent on cellular energy.

The altered sensitivity of the basidiome inhibitory mutants to antibiotics may be related to defective membrane bound proteins which are known to affect cell membrane permeability. *E. coli* and *P. aeruginosa* strains which lack porin proteins are more resistant than wild type

strains to hydrophilic antibiotics, particularly β-lactams (Harder *et al.* 1981, Woodruff & Hancock 1988).

Availability of iron is known to affect secondary metabolite production in many organisms (Neilands 1984). In *P. putida* availability of iron regulates production of cyanide (Bakker & Schippers 1987), in *P. syringae* pv. *syringae* iron regulates production of syringomycin (Cody & Gross 1987). Iron also regulates the production of a range of membrane bound proteins in bacteria (Neilands 1982, de Weger *et al.* 1986). While some of these proteins play a role in iron aquisition (see above), the role of others has not been determined, but it is likely that they are important in other cellular processes. Mutations which affect the aquisition of iron by *P. putida* are therefore likely to affect other cellular activities, which in turn may have an effect on the ability of *P. putida* to stimulate basidiome initiation. Consideration of the regulatory role of iron is necessary when trying to link mutants defective in their ability to aquire iron, with their inability to promote basidiome initiation.

Scarcity of basidiome inhibitory mutants

The failure to generate further basidiome inhibitory mutants at Canterbury University, despite repeated attempts, was surprising. At I.H.R., UV light proved an efficient means of producing non-fluorescent mutants and the high frequency encountered on some occasions suggested the presence of a mutational hot-spot. Why this was not found at Canterbury University is not known, but may have been due to differences in the UV light source. Transposon mutagenesis at Canterbury University also failed to produce any basidiome inhibitory mutants, however, as it appears that it was necessary to inactivate more than a single gene in order to create the desired mutant, this was not unexpected. Transposon mutants usually possess only a single defective gene, although mutants apparently defective in several genes may arise if the transposon inserts within a regulatory gene or if the cell acquires more than one Tn*5* insertion. It is possible that the Tn*5* generated basidiome inhibitory mutant was a 'lucky find' and that the insertion was within a major regulatory gene into which Tn*5* does not ordinarily insert. Sokol (1987) was unable to isolate, from 5000 Tn*5* mutants, any which were completely negative in expression of the ferripyochelin-binding (FPB) outer membrane protein.

The Tn5 generated basidiome inhibitory mutants, isolated after screening directly against W19, all grew poorly in the presence of the fungus. This suggests that they were more sensitive to antimicrobial compounds produced by W19 (see 4.4.2.) than the wild type strain. Increased sensitivity to antimicrobial compounds probably resulted from alterations in cell wall permeability which further indicates that mutations affecting the bacterial cell wall, affect the ability of *P. putida* to promote basidiome initiation. The production of an atypical siderophore by one of these mutants also indicated that the bacterial iron scavenging system is in some way involved in the process of basidiome initiation.

During an experiment aimed at examining the growth of three colony forms of *P. putida* PMS118 (PMS118S, PMS118R and PMS118Rr) under nutrient limited conditions (see Chapter 2.0.), 98 % of the colonies, in three separate flasks, were found to be non-fluorescent after 4 h growth in dilute KB broth. This decreased to 50 % after 8 h and 0 % after 18 h. Non-fluorescent cells from each flask were kept and all stably maintained this phenotype, in addition, all inhibited basidiome initiation. One of these mutant, PR225, was examined and possessed biochemical and nutritional characteristics consistent with *P. putida* (see Chapter 2.0.). This result suggests that a number of genes involved in the biosysnthesis and uptake of siderophores can be switched off, or lost.

This study has revealed the complexity of the interaction between *P. putida* and *A. bisporus* at a genetic level. There is much scope for further work which should be initially directed toward generation of more basidiome inhibitory mutants and should aim to fully characterize the available mutants. Mutant selection should be made by screening further Tn5 and UV light generated mutants directly against W19 rather than selecting for non-fluorescent mutants. It would also be beneficial to try and determine accurately, the precise effect of the mutants on the fungus.

Comments on the siderophore mutants

The siderophore mutants characterized in this study are similar to those described by Marugg *et al.* (1985). These workers described six mutant classes which enabled them to propose a

biosynthetic pathway for the *P. putida* siderophore (see section 2.3.4.). In *P. putida* WCS358 this is thought to begin with synthesis of the peptide part of the molecule, followed by synthesis, in several steps, of the fluorescing group; both parts of which have been shown to be involved in ferric binding (Teintze *et al.* 1981). The *P. putida* PMS118S mutants suggest that a similar biosynthetic pathway operates in this organism.

Class 1 mutants did not fluoresce, but still bound iron efficiently indicating that some alterations in the fluorescing part of the molecule do not effect the binding or uptake process. The class 2 mutants were unable to produce a fluorescent pigment, or sequester iron and as suggested by Marugg *et al.* (1985), are probably blocked at an earlier stage in the biosynthetic process. This indicates that the fluorescing group is not synthesized independently from the rest of the molecule and occurs at a later stage of the biosynthesis of the complete molecule (Marugg *et al.* 1985). Mutants of classes 5, 6, 7 and 8, which possess intermediate phenotypes, were probably mutated in a regulation step, or in the excretion process. Mutants of classes 3 and 4 were unable to uptake the ferric-siderophore (see above). Cells defective in their ability to uptake ferric-siderophore were not detected by Marugg *et al.* (1985), or Moores *et al.* (1984), because of the method they employed to detect siderophore mutants. Uptake mutants were, however, found by Cody & Gross (1987) in *P. syringae* pv *syringae* and by Sokol (1987). Class 4 mutants produced a functional siderophore which indicates that mutations affecting ferric-siderophore uptake do not affect production of the siderophore. This is consistent with the knowledge that in *P. putida*, genes encoding for the uptake system are located on a different operon to the biosynthetic genes (Magazin *et al.* 1986). The class 3 mutant was particularly unusual and the loss of fluorescence, siderophore production and ability to uptake the ferric-siderophore indicates that a major regulatory gene is probably affected (see above). Class 3 and 4 mutants should prove useful for subsequent studies on the uptake of the ferric-siderophore complex by *P. putida*. The ability of class 3 & 4 mutants to grow on KB also demonstrates the existence of a low affinity pathway for the aquisition of iron.

5.4.2. RP4::MINI-MU GENE CLONING AND MAPPING IN *P. PUTIDA*

Despite the problems initially encountered with instability of the RP4::mini-Mu plasmids, the ability of plasmid pULB113 to transfer to *P. putida* and promote efficiently the transfer of chromosomal markers in homologous and heterologous matings was shown. Previously, plasmid pULB113 was found to promote chromosome mobilization and R-prime formation in various enteric bacteria and Lejeune *et al.* (1983) demonstrated these properties in two species unrelated to the enteric bacteria; *Alcaligenes eutrophus* and *P. fluorescens*. This work further demonstrates the applicability and potential of this *in vivo* cloning system for non-enteric bacteria. Transfer of genetic markers occurred at frequencies ranging from 10^{-5} to 10^{-8} per recipient cell, which are comparable to the frequencies found in enteric bacteria (Van Gijsegem & Toussaint 1982, Lejeune *et al.* 1983) and *A. eutrophus* and *P. fluorescens* (Lejeune *et al.* 1983). As a result of the lack of suitable mutants, chromosome mobilization was not unequivocally proven, nevertheless, there was no evidence of R-prime formation following homologous matings in recombination proficient backgrounds, which strongly indicates transfer of markers by chromosome mobilization. This trait is useful for genome mapping and strain construction.

Formation of R-prime plasmids was shown in heterospecific matings from *P. putida* to *E. coli*, however, these plasmids were unstable in *E. coli*. Poor expression and instability of *Pseudomonas* genes in *E. coli* has been previously reported (Jeenes *et al.* 1986, Nakazawa & Inouye 1986, Marugg *et al.* 1988) and demonstrates the need to develop well defined, *Pseudomonas* derived, host-vector systems.

The development of a Rec$^-$ strain (PR118) was a step this direction and is most likely necessary for R-prime formation in homologous matings between *P. putida* strains. Unfortunately, because of the difficulties encountered with complementing Tn*5* generated auxotrophs using pULB113, it was not possible to determine the extent of recombinational deficiency of *P. putida* PR118. Nevertheless, as all cells with impaired recombinational proficiencies are sensitive to UV light (Fruh *et al.* 1983, Miller 1972, Clark & Margulies 1965, Smith 1988, Walker 1984), it would seem reasonable to assume that recombination in PR118 is

impaired. It would be worthwhile to characterize this strain further and to determine the extent of the recombinational mutation.

Not all selected genes were complemented in heterospecific crosses. The Pro auxotroph of *E. coli* HB101 was not complemented by Pro from either *P. putida* PMS118S or PMS196, and PMS118S did not complement the *P. aeruginosa* OT11 Pro auxotroph, however, Pro from PMS118S complemented the Pro mutation in *E. coli* RR1. This suggests that there are differences in the pathways used by *P. putida*, *P. aeruginosa* and *E. coli*, to metabolize proline. The ability of *P. putida* PMS118S to complement the Pro *E. coli* RR1 mutants is difficult to explain, especially as both *E. coli* strains are reportedly mutated in the same allele; *pro*A2. Proline utilization by *P. putida* has not been examined in detail and therefore it is not possible to determine the nature of these differences.

The inability of *P. putida* to complement the *arg*32 mutation in *P. aeruginosa* indicates that *P. putida* uses a different pathway for the metabolism of this amino acid. Three main pathways for arginine utilization in *Pseudomonas* are known and the genus is known to be heterogeneous with respect to the pathways employed (Phillips 1986).

This work has presented evidence that pULB113 is able to promote both homologous and heterologous gene transfer in *P. putida* and demonstrates the potential of this plasmid for genetic manipulations in this organism. While the RP4::mini-Mu gene cloning and mapping system requires further investigation, a large part of the fundamental ground work has been performed and it should be possible to use this system to clone and map *P. putida* genes involved in a variety of processes, including basidiome initiation of *A. bisporus*.

5.4.3. MARKING AND MOBILIZATION OF A CRYPTIC *P. PUTIDA* PLASMID

The suitability of the Tn*5*-Mob system for marking and mobilizing cryptic plasmids in *Pseudomonas* was demonstrated. Plasmid pPU1 did not confer on *E. coli*, or *Serratia entomophila*, the ability to promote basidiome initiation, but this does not demonstrate the non-involvement of this plasmid in fruiting. Transfer of pPU1 into a basidiome inhibitory *P. putida* mutant would provide a more certain indication of the involvement of this vector in fruiting. By

performing matings between PMS118S and PR220(pPU1) it should be possible to introduce Tn5-Mob into pPU1 in the original host strain. It would then be possible to cured the plasmid from PMS118S and examine its effect on basidiome initiation.

The smaller *P. putida* PMS118S plasmid, pPU2, was not marked and mobilized, probably because it did not receive Tn*5*-Mob. Selection of a greater number of transconjuants (ca. 200) would probably be needed to ensure at least one clone has received the transposon inserted in pPU2. The instability of pPU1::Tn*5*-Mob in *E. coli* HB101 recipients suggests that this plasmid could be useful as a suicide vector for the introduction of Tn*5* into the chromosome of *E. coli* and other Gram-negative bacteria unrelated to *P. putida* (Simon 1984).

CHAPTER SIX

CONCLUSION

6.1. CONCLUSION

The taxonomy of the pseudomonads used throughout the study was investigated and the phenomenon of colony morphogenesis examined. The prodigious morphogenetical capabilities of *P. putida* and *P. tolaasii* were demonstrated and as suggested by Shapiro (1986), probably represent the expression of basic functions for cellular differentiation and the organization of large populations. Rough colonial forms were shown to arise from smooth colonial variants in peat casing soil indicating that rough forms are not an artifact of *in vitro* cultivation. Rough colonial forms of *P. putida* were shown to be capable of promoting basidiome initiation.

Investigations into physiological aspects of the different colonial forms indicated that the rough forms were better adapted to withstand harsh conditions. These stress-tolerant forms may be important in the competitive casing layer environment where nutrients are scarce. Taxonomic differences between rough and smooth colonial forms of a single strain were shown to be, in some cases, at least as great as the differences between closely related species. This has implications for pseudomonad taxonomy and requires further consideration.

P. putida (and *P. tolaasii*) was positively attracted toward exudates of mushroom mycelium and shown to adhere rapidly and firmly to hyphal surfaces. Adherence was found to be markedly affected by the medium on which the bacterium was cultured and was greatest when grown on a substrate resembling the nutrient status of the casing layer. Polysaccharide material and electrostatic forces appeared to be responsible for enabling rapid, firm attachment to occur.

Two major problems which have previously hampered studies aimed at investigating fruiting of *A. bisporus*; the lack of a suitable medium for the growth of the fungus and lack of a bioassay for determining the effect of bacteria on basidiome initiation, were resolved. CMM provided a solution to the first problem and has proved an invaluable laboratory substrate, promoting rapid and vigorous mycelial growth of *A. bisporus* and associated bacteria. The discovery of strain W19 and development of the simple *in vitro* fruiting system provided a solution to the second problem and has enabled work on the role of bacteria in fruit body initiation of *A. bisporus* to proceed rapidly. Investigations into the nature of the microbial stimulus effecting

basidiome initiation demonstrated the requirement for living *P. putida* and showed that transition from a nutrient rich to a nutrient poor substrate was not sufficient to promote fruiting. Previous work had shown that activated charcoal could replace the effect of micro-organisms and this was confirmed. The work of Wood (1976), which discounted a role for iron and iron chelating agents in fruiting, was also confirmed.

The ability to delimit stimulation of mycelial growth by *P. putida* from primordia initiation, by culturing *A. bisporus* and associated bacteria on CMM, provided an opportunity to examine, in a manageable system, the initial stage of the fruiting response. Results gained from this investigation were consistent with previous findings which indicated that stimulation of mycelial growth represents a preliminary event in the process of basidiome morphogenesis.

Mutants of *P. putida* were generated by a range of methods and a number were isolated which were unable to promote basidiome initiation. Characterization of these mutants indicated that genes responsible for biosynthesis of the siderophore and uptake of the ferric-siderophore complex were involved in triggering fruiting. A role for the iron regulated outer membrane protein porins was envisaged:- It is possible that these porins are responsible for uptake of the proposed 'self-inhibitory-compound' and when non-functional, fruiting is inhibited as a result of high levels of the 'self-inhibitory-compound'.

The next stage of genetic analysis, following the production of further basidiome inhibitory mutants, involves isolation, cloning and mapping of the appropriate genes. The RP4::mini-Mu *in vivo* gene cloning and mapping system was investigated for this purpose and the potential of this system for cloning and mapping genes in *P. putida* was demonstrated.

6.1.1. A MODEL FOR BASIDIOME INITIATION OF *A. BISPORUS*

The work presented in this thesis confirms the existence of a unique and novel relationship between *A. bisporus* and *P. putida*. The following model is put forward to explain the fruiting process and the role of *P. putida*.

Prior to fruiting, *A. bisporus* establishes itself within a given environment and exploits a nutrient pool of sufficient size to support mycelial proliferation and fruit body production. Having done this, the fungus becomes 'receptive' to the presence of basidiome stimulatory *P. putida*

(this bacterium is found in large numbers within the mushroom casing layer and would most likely occur in all environments where *A. bisporus* can grow). Bacteria are attracted to low molecular weight compounds exuded by the fungus, most likely simple carbohydrates and amino acids, and migrate toward the hyphae and adhere to the surfaces. Following attachment, the bacteria multiply at the expense of nutrients exuded by the hyphae and begin to import, through the iron regulated outer membrane bound protein porins, the proposed 'self-inhibitory-compound'. This triggers the onset of reproductive growth. During the initial stage of fruiting, the rate of mycelial extension increases and the fungus directs growth toward regions of the substrate where *P. putida* exists (particularly the surface of the soil or casing layer - pseudomonads are obligate aerobes) and growth is restricted over areas where the bacterium does not exist. This ensures that the fungus colonizes as large an area as possible over which the reproductive stimulus is present, enabling the fungus to maximize the number of fruit bodies produced for a given resource unit. As the number of bacteria colonizing the hyphal surfaces increases, the amount of 'self-inhibiting-compound' is reduced until the concentration of this compound, in the immediate vicinity of the hyphae, has been reduced to below a threshold, enabling basidiome initiation to take place (Fig. 6.1).

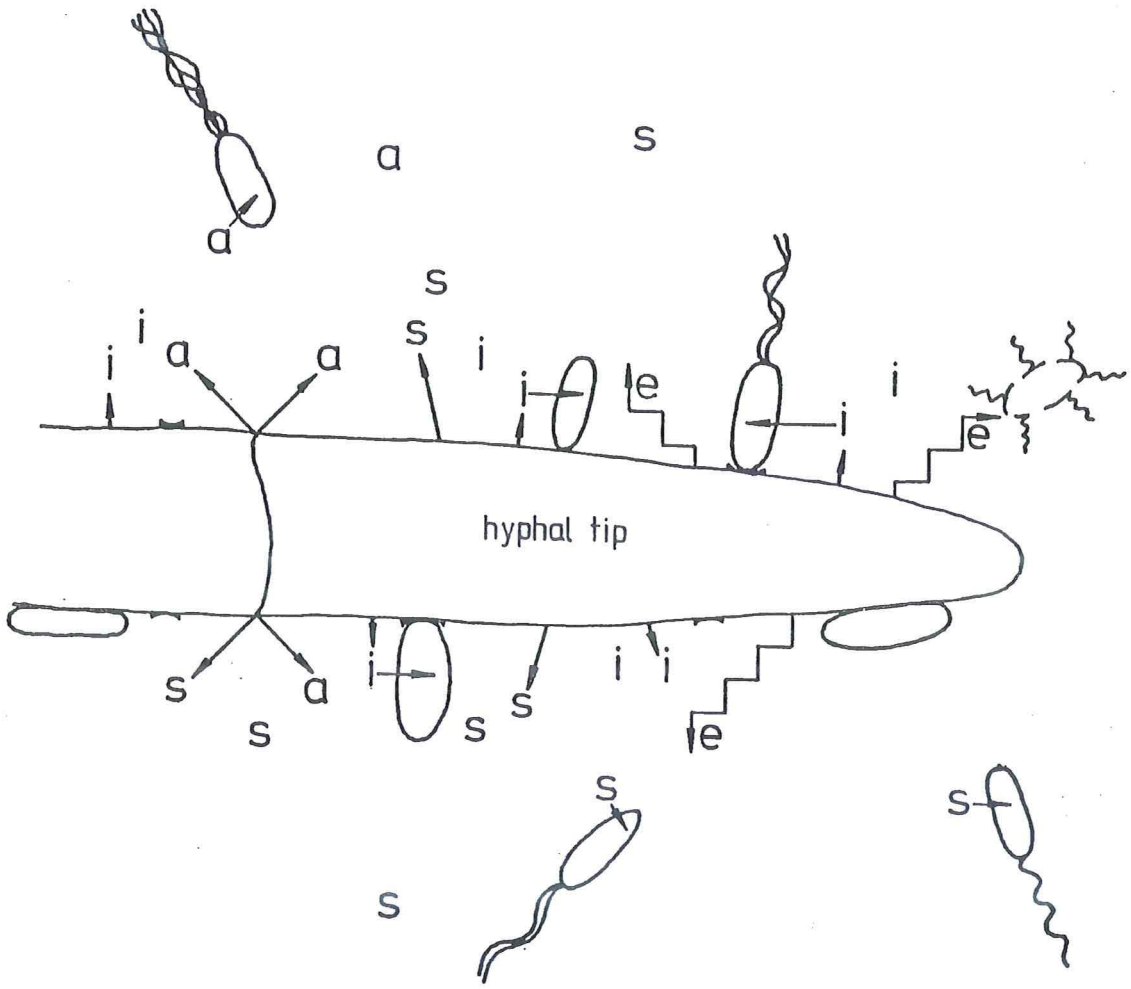


Fig. 6.1. Proposed model for the involvement of *P. putida* in the process of basidiome initiation of *A. bisporus*. Diagram shows a hyphal tip exuding nutrients (possibly amino acids (**a**) and simple sugars (**s**)) which serve as attractants for the bacteria. *P. putida* resists the degradative enzymes (**e**) secreted by the fungus and adheres to the hyphal surface, possibly after recognizing specific receptors. Following attachment, the bacteria import (through the iron regulated membrane bound proteins) and metabolize the proposed 'self-inhibitory-compound' (**i**), reducing the concentration of this compound to below a threshold level which triggers basidiome initiation.

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APPENDIX A: MEDIA

Reagents are for 1 l; agar was omitted for broth cultures

King's Medium A (King *et al.* 1954)

Bacto peptone	20 g
Bacto agar	15 g
Glycerol	10 g
K_2SO_4	10 g
$MgCl_2$	1.4 g
Agar	15 g
pH 7.2	

King's Medium B (King *et al.* 1954)

Proteose peptone No. 3	20 g
Glycerol	10 g
K_2HPO_4	1.5 g
$MgSO_4.7H_2O$	1.5 g
Agar	15 g
pH 7.2	

Luria (L) agar (Miller 1972)

Bacto-tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Agar	15 g
pH 7.5	

Minimal M9 medium (Maniatis 1982)

Na_2HPO_4	6 g
KH_2PO_4	3 g
NaCl	0.5 g
NH_4Cl	0.5 g
Agar (autoclaved separately)	15.0 g

Add to the cooled, sterile salt solution: 1 ml of 20 % MgSO4.7H2O (filter sterilized), 0.5 ml of 1 % thiamime (filter sterilized), and 10 ml of 20 % glucose (filter sterilized).

Minimal M63 medium (Miller 1972)

KH_2PO_4	13.6 g
$(NH_4)_2SO_4$	2.0 g
$FeSO_4.7H_2O$	1.25 mg
pH 7.0 with 5 N KOH	
Agar (autoclaved separately)	15.0 g

Add to the cooled, sterile salt solution: 1 ml of 20 % $MgSO_4.7H_2O$ (filter sterilized), 0.5 ml of 1 % thiamime (filter sterilized), and 10 ml of 20 % glucose (filter sterilized).

Nutrient agar - yeast extract

Bacto Nutrient agar	23 g
Yeast extract	3 g

Pseudomonas **agar F (Difco)**

Proteose peptone No. 3	10 g
Bacto tryptone	10 g
Glycerol	10 g
K_2HPO_4	1.5 g
$MgSO_4.7H_2O$	1.5 g
Agar	15 g
pH 7.2	

Succinate salts medium (Meyer & Abdallah 1978)

K_2HPO_4	6.0 g
KH_2PO_4	3.0 g
$(NH4)_2SO_4$	1.0 g
$MgSO_4.7H_2O$	0.2 g
Succinate acid	4.0 g
pH 7.0	

Tripticase soy broth agar (Miller & Berger 1985)

Tripticase soy broth	30 g
Agar	15 g

APPENDIX B: BUFFERS

Reagents are for 1 l.

Phosphate Buffered Saline (PBS) (Matthysse *et al.* 1978)

Na_2HPO_4	7 g
KH_2PO_4	3 g
NaCl	4 g
$MgSO_4.7H_2O$	0.2 g

Phosphate Urea Magnesium buffer (PUM) (Rosenberg *et al.* 1980)

K_2HPO_4	16.87 g
KH_2PO_4	7.26 g
$MgSO_4.7H_2O$	0.2 g
Urea	1.8 g

Sodium Acetate Buffer (Carlton & Brown 1981)

$NaC_2H_3O_2.3H_2O$	13.6 g

Adjust pH to 4.6 with glacial acetic acid

Alkaline SDS solution (Birnboim & Doly 1979)

0.2 N NaOH
1 % sodium dodecyl sulphate (SDS)

DNA Sample buffer (Maniatas 1982)

0.25 % bromophenol blue
0.25 % xylene cyanol
30 % glycerol

High salt solution (Birnboim & Doly 1979)

3 M sodium acetate (pH 4.8) (adjusted with glacial acetic acid)

Lysis buffer (Birnboim & Doly 1979)

1 mg ml^{-1} lysozyme
50 mM glucose
10 mM EDTA
25 mM Tris-HCl (pH 8.0)

Neutralization buffer (Birnboim & Doly 1979)

0.1 M sodium acetate
0.05 M Tris-HCl (pH 8.0)

Tris acetate gel running buffer (Maniatis 1982)

40 mM Tris acetate
2 mM EDTA (pH 8.0)

APPENDIX C: PLASMIDS IN PSEUDOMONAD ISOLATES

Plasmid profiles of pseudomonads isolated from the casing layer are shown in Figs C.1 and C.2. The plasmid DNA was prepared according to the methods in section 5.2.4. and plasmid size was estimated by the procedure outlined in Maniatis (1982). Plasmid standards; FP2, RP4 and pSAC3, were used (see Table 5.1).

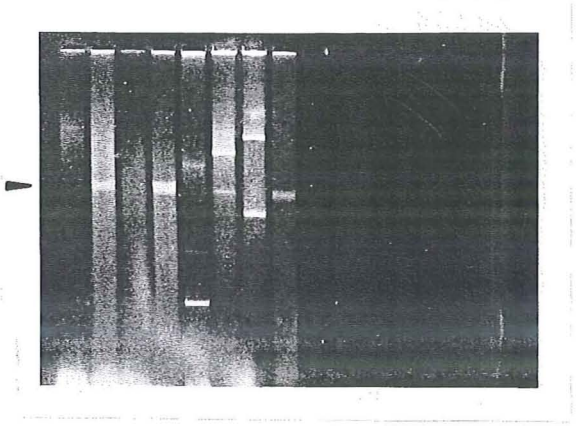

Fig. C.1. Electropherogram of plasmid DNA extracted from pseudomonad isolates. Left to right; FP2 (90 kb); *Pseudomonas* sp., no plasmid; *Pseudomonas* sp., no plasmid; *Pseudomonas* sp, plasmid > 100 kb; pSAC3 (7.6 kb), RP4 (60 kb), *P. putida* PMS118S, plasmid pPU1 ca. 80 kb and pPU2 ca. 24 kb), *Pseudomonas* sp., no plasmid. Arrow shows chromosomal DNA.

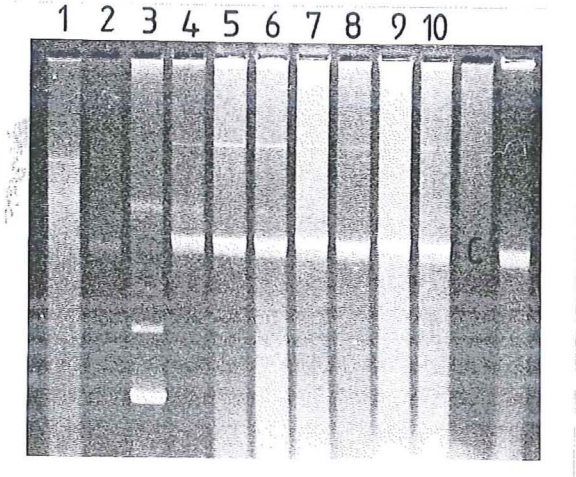

Fig. C.2. Electropherogram of plasmid DNA extracted from smooth and rough colonial froms of *P. tolaasii* PMS117. Lanes 1 and 2, FP2 (90 kb); lane 3, pSAC3 (7.6 kb); lanes 4 - 7 PMS117R (plasmid > 100 kb); lanes 8 - 9 PMS117S (plasmid > 100 kb). C shows chromosomal DNA.